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(54) Title: A NOVEL SPECIFIC INHIBITOR OF THE CYCLIN KINASE INHIBITOR P21$^{WAF1/CIP1}$

(57) Abstract: Methods and compositions for regulating abnormal cell growth and proliferation mediated by p21$^{Waf1/Cip1}$ using inhibitors of p21$^{Waf1/Cip1}$.

# A NOVEL SPECIFIC INHIBITOR OF THE CYCLIN KINASE INHIBITOR p21 Wafl/Cip1

This application is based on and claims the priority of U.S. Serial Number 60/193,155, filed March 29, 2000, the contents of which are hereby incorporated by reference in their entirety.

Throughout this application various publications are referenced. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application, in order to more fully describe the state of the art to which this invention pertains.

## FIELD OF INVENTION

The present invention relates to methods and compositions for regulating cell growth and proliferation, mediated by cyclin-dependent kinases, by inhibiting $p21^{Waf1/Cip1}$, and more particularly to the prevention and treatment of diseases associated with abnormal proliferation of cells, using $p21^{Waf1/Cip1}$ inhibitory agents.

## BACKGROUND OF THE INVENTION

The mechanism by which cells "decide" whether to grow or not to grow is of paramount importance in a surprising variety of diseases. Unregulated or abnormal cell growth in vascular smooth muscle (VSM) and phenotypically-related glomerular mesangial cells (Dubey RK, et al. *Curr Opin Nephrol Hypertens* 6:88-105, 1997), is the underlying pathogenic mechanism in such diverse diseases as hemodialysis graft stenosis, angioplasty restenosis (Ross, 1993 *Nature*, 362, p. 801-809), and atherosclerosis, as well as in mesangial proliferative kidney disease (Megyesi et al., 1999 *Proc. Natl. Acad. Sci. U.S.A.*, 96, p. 10830-10835). It has been suggested that pharmacological methods to increase $p21^{Waf1/Cip1}$ may be useful in preventing the VSM cell proliferation seen after coronary angioplasty (Kusama et al., 1999 *Atherosclerosis*, 143, p. 307-313; Takahashi et

al., 1999 *Circ. Res.*, 84, p. 543-550; and Yang et al., 1996: *Semin. Interv. Cardiol.*, 1, p. 181-184). Thus, research directed at understanding the mechanisms by which these processes occur in VSM cells is critical to the development of specific therapies for these diseases.

Most likely due to the fact that protection from these often deadly diseases has provided significant survival advantage over evolutionary time, organisms have evolved complex and often redundant systems for keeping these essential, but potentially lethal, cellular processes, such as unregulated cell growth, in check. Consequently, there exist cell cycle regulators at multiple levels of the cell growth hierarchy: from the growth factor receptor regulatory proteins (such as receptor phosphorylation events and various G-proteins), through the cytoplasmic signal protein interactions (such as the mitogen-activated protein serine/threonine kinase (MAPK), stress-activated protein kinase (SAPK), and Janus family of protein kinase-signal transducers and activators of transcription (JAK-STAT) systems [Frye, R.A. in *Oncogenes and Tumor Suppressor Genes in Human Malignancies*, Benz CC and Liu, ET, eds. 63:281-299, Kluwer Academic Publishers, Boston], to the nuclear transcriptional control machinery (such as the cyclins, cyclin kinases, and cyclin kinase inhibitors) (Lavoie JN, et al. *Prog Cell Cycle Res* 2:49-58, 1996).

Mechanisms and regulation of the cyclin kinase system in VSM cells have been studied. These molecules (such as the cyclins, cyclin kinases, and cyclin kinase inhibitors), which regulate cell growth, are very distal along the growth factor signaling pathways, and may, therefore, be among the ultimate arbiters of the decision a cell must make whether it will proceed through the cell cycle and lead to the production of cellular progeny or die. Since many growth-controlling signaling pathways converge on the cyclin system, elucidating the mechanism of regulation of these molecules will likely lead to the development of pharmaceuticals which target these molecules and, consequently, are useful for the treatment of vascular and renal diseases as well as cancer.

Cell cycle progression is finely regulated by the interplay between the cyclin-dependent kinases (cdks) and the cdk inhibitors (CKIs). Cyclin is a protein involved in the cell cycle that accumulates during interphase and is destroyed during mitosis. Cdks are a well-conserved family of serine/threonine protein kinases, found in yeast and in at least eight different animal cells, which function in mitogenic signaling through their activation by the cyclins. This in turn leads to a cascade of events whereby the mitogen-stimulated cyclin D-dependent kinases phosphorylate retinoblastoma protein (Rb), causing release of inhibition of the transcription factor family known as E2F, and allowing S-phase specific gene transcription and subsequent progression through the G1/S transition (Sherr and Roberts, 1999, *Genes and Dev.*, 13, p. 1501-1512).

The Cip/Kip family of CKIs ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) regulate the activity of the cyclin/cdk complex and have been shown to negatively regulate the process of cyclin-mediated cell cycle progression through inhibition of the cdks ($p21^{Waf1/Cip1}$ (Gu et al. 1993, *Nature*, 366, 707-710; Harper et al., 1993, *Cell*, 75, 387-400; El-Deiry et al. 1993, *Cell*, 75, 817-825; Xiong et al. 1993, *Nature*, 366, 701-704; Dulic et al. 1994, *Cell*, 76, 1013-1023; Noda et al. 1994, *Exp. Cell Res.*, 211, 90-98), $p27^{Kip1}$ (Polyak et al. 1994, *Genes & Dev.* 8, 9-22; Polyak et al. 1994, *Cell*, 78, 59-66; and Toyoshima and Hunter, 1994, *Cell*, 78, 67-74), and $p57^{Kip2}$ (Lee et al. 1995, *Genes & Dev.* 9, 639-649; and Matsuoka et al. 1995, *Genes & Dev.* 9, 650-662)).

The protein $p21^{Waf1/Cip1}$ was first described in 1992 (Xiong et al., 1992 *Cell*, 71, p. 505-514). The sequences of the human, rat and mouse $p21^{Waf1/Cip1}$ genes are known (GenBank entries CAB06656, I84725 and I49023, respectively), and polyclonal and monoclonal antibodies, to human and rodent species, are commercially available. The human protein has been expressed in *E. coli* by commercial sources (Santa Cruz Biotechnology, Santa Cruz, CA).

The net result of induction or overexpression of the CKIs (particularly those in the Cip/Kip family) generally is cell cycle inhibition and growth suppression in VSM and other cell types (Chang et al., 1995 *J. Clin. Invest.*, 96, p. 2260-2268; Ishida et al., 1997

*J.Biol.Chem.*, 272, p. 10050-10057; Matsushita et al., 1998 *Hypertension*, 31, p. 493-498; Sewing et al., 1997 *Mol. Cell Biol.*, 17, p. 5588-5597; and Weiss et al., 1999 *J. Am. Soc. Nephrol.*, 9, p. 1880-1890). Consistent with this, the CKIs are down-regulated in response to a variety of mitogens, and overexpression of these molecules leads to growth arrest (Kato et al., 1994 *Cell*, 79, p. 487-496; Nourse et al., 1994 *Nature*, 372, p. 570-573; Pagano et al., 1995 *Science*, 269, p. 682-685; and Resnitzky et al., 1995 *Mol. Cell Biol.*, 15, p. 4347-4352).

While much of the early work on the CKIs has focused on their role as growth inhibitors, it had been somewhat puzzling that expression of these molecules increases early after mitogen stimulation (Depoortere et al., 1996 *J. Cell Sci.*, 109 (Pt 7), p. 1759-1764; and Michieli et al., 1994 *Cancer Res.*, 54, p. 3391-3395). CKIs have also been implicated in positive effects on cyclin/cdk activitation (Cheng et al., 1998 *Proc. Natl. Acad. Sci.U.S.A.*, 95, p. 1091-1096; LaBaer et al., 1997 *Genes Dev.*, 11, p. 847-862; and Zhang et al., 1994 *Genes Dev.*, 8, p. 1750-1758). This led to more recent data showing the ability of some CKIs to take part in formation of the cyclin/cdk complexes, and thus to serve as "assembly factors" important for promoting cyclin/cdk association (Hiyama et al., 1998 *Oncogene*, 16, p. 1513-1523; and LaBaer et al., 1997 *Genes Dev.*, 11, p. 847-862). Various CKIs have also been reported to act as "assembly factors" in other cells, both *in vivo* (Cheng et al., 1999 *EMBO J.*, 18, p. 1571-1583) and *in vitro* (LaBaer et al., 1997 : *Genes Dev.*, 11, p. 847-862). In support of this role for the CKIs, others have shown that assembly of cyclin D1/D2-cdk4 complexes was impaired in fibroblasts from mice lacking the $p21^{Waf1/Cip1}$ and/or $p27^{Kip1}$ genes (Cheng et al., 1999 *EMBO J.*, 18, p. 1571-1583), and that both $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ actively promoted interaction between the cyclin Ds and their counterpart cdks by stabilizing this complex (LaBaer et al., 1997 *Genes Dev.*, 11, p. 847-862). However, primary fibroblasts from p21- and p27-null mice did not show overtly abnormal cell cycles, despite the finding by those investigators that overall cyclin D-dependent kinase activity was reduced below the assay limit of detectability. Previous studies have not shown inhibition of growth with interference of cyclin/cdk association. The cyclin D1/cdk 4 interaction occurs early after growth factor stimulation (reviewed in Arellano and Moreno, 1997 *Int. J. Biochem. Cell Biol.*, 29, p.

559-573) and this interaction is facilitated by $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ *in vivo* (LaBaer et al., 1997: *Genes Dev.*, 11, p. 847-862).

Thus, CKIs exhibit both positive and negative effects on growth and apoptosis in a variety of cell types, including VSM cells. A further example of this is the mechanism of action of the HMG CoA reductase inhibitors, where accelerated graft atherosclerosis in heart, and probably renal, transplant patients is attenuated by the statins (Katznelson S, et al. *Transplantation* 61:1469-1474, 1996; and Southworth MR, Mauro VF. *Ann Pharmacol* 31:489-491, 1997). Many of the statins have been shown to attenuate smooth muscle growth and promote apoptosis in association with an increase in the cyclin kinase inhibitors p21 and p27 (Baetta R, et al. *Pharmacol Res* 36:115-121, 1997; Terada Y, et al. *J Am. Soc Nephrol* 9:2235-2243, 1999; Weiss RH, et al. *J Am Soc Nephrol* 9:1880-1890, 1999; and Laufs U, et al. *J Biol Chem* 274:21926-21931, 1999), although whether this is the mechanism of this effect is unknown. Tumor cells that are p21(-/-) are also known to be sensitized to apoptosis (Stewart ZA, et al. *Cancer Res* 59:3831-3837, 1999; Fan S et al. *Oncogene* 14:2127-2136, 1997). What constitutes the "switch" from positive to negative effects of the cyclin kinase inhibitors on both growth and apoptosis is unclear.

There is a need for improved therapies of diseases, associated with abnormal cell growth and proliferation, that take into account the various pathways that result in stimulation of growth and of cells. For the first time, it is shown in the present invention that $p21^{Waf1/Cip1}$ serves a permissive role in platelet-derived growth factor (PDGF)-mediated VSM cell proliferation, such that its presence is required for the mitogenic effect of PDGF. It is thus possible to devise therapeutic strategies to inhibit cell proliferation, in proliferative diseases, by controlling the expression of CKIs, in particular $p21^{Waf1/Cip1}$.

## SUMMARY OF THE INVENTION

Accordingly, the present invention provides novel methods and compositions for regulating cell growth and proliferation, and treating diseases associated with abnormal cell growth and proliferation, mediated by cyclin-dependent kinases, by inhibiting

$p21^{Waf1/Cip1}$ in cells expressing $p21^{Waf1/Cip1}$, using $p21^{Waf1/Cip1}$ inhibitory agents. The methods are also for preventing and treating fibrotic diseases associated with abnormal cell growth and proliferation. The methods, further include, inhibiting angiogenesis and tumor growth by inhibiting $p21^{Waf1/Cip1}$ in cells expressing $p21^{Waf1/Cip1}$, using $p21^{Waf1/Cip1}$ inhibitory agents. The methods include using inhibitory agents such as an antisense oligonucleotide of $p21^{Waf1/Cip1}$ and anti-$p21^{Waf1/Cip1}$ antibodies, to prevent transcription and expression of $p21^{Waf1/Cip1}$.

The therapeutic methods of the invention can also be used in conjunction with radiation therapy and chemotherapy.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-D are bar graphs showing that antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide transfection inhibits VSM cell DNA synthesis in a dose-dependent manner, as described in Example I, *infra*. A10 VSM cells were lipofected with (A) no DNA, 200 nM of sense $p21^{Waf1/Cip1}$ or antisense $p21^{Waf1/Cip1}$; and (B) 200 nM of random sequence control oligodeoxynucleotide or antisense $p21^{Waf1/Cip1}$; and various concentrations of sense $p21^{Waf1/Cip1}$ or antisense $p21^{Waf1/Cip1}$ in (C) A10 and (D) bovine VSM cells. The experiments shown are representative of two to three separate experiments.

Figure 2 is a bar graph showing VSM cell proliferation is inhibited by antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide, as described in Example I, *infra*. A10 VSM cells were transfected as in Figure 1C. Cell numbers are expressed as mean ± s.e.m. of three wells per data point.

Figure 3 A and B show photographs of antisense oligodeoxynucleotides which were successfully transfected into VSM cells, as described in Example I, *infra*. FITC-tagged $p21^{Waf1/Cip1}$ antisense oligodeoxynucleotide was lipofected into A10 VSM cells and the same microscopic field was visualized by (A) visible and (B) fluorescence light at 40x.

Figure 4 depicts a gel showing PMA induces $p21^{Waf1/Cip1}$ in VSM cells, as described in Example I, *infra*. Non-transfected A10 VSM cells were stimulated with PMA (100 ng/ml) for the times indicated and Western blotted with $p21^{Waf1/Cip1}$ antibody. The experiment shown is representative of three separate experiments.

Figure 5 depicts a gel showing PMA stimulated CKI induction is blunted in antisense $p21^{Waf1/Cip1}$ transfected cells, as described in Example I, *infra*. A10 VSM cells were transfected with antisense $p21^{Waf1/Cip1}$ or sense $p21^{Waf1/Cip1}$ oligodeoxynucleotide as in Figure 1. After overnight incubation, the cells were stimulated with PMA for the times indicated, lysed, and the lysates were Western blotted with $p21^{Waf1/Cip1}$ antibody. The experiment shown is representative of three separate experiments.

Figure 6 A and B are gels showing antisense $p21^{Waf1/Cip1}$ inhibition of $p21^{Waf1/Cip1}$, as described in Example I, *infra*. A10 VSM Cells were lipofected with antisense $p21^{Waf1/Cip1}$ or sense $p21^{Waf1/Cip1}$ oligonucleotides, as in Figure 1. (A) antibody to $p21^{Waf1/Cip1}$; antibody to $p27^{Kip1}$ (B) α-actin antibody. The experiments shown are each representative of two separate experiments.

Figure 7 depicts a gel showing antisense $p21^{Waf1/Cip1}$ inhibits cyclin D1/cdk4, but not cyclin E/cdk2, association, as described in Example I, *infra*. A10 VSM cells were transfected with antisense or sense $p21^{Waf1/Cip1}$ as in Figure 1. The arrowhead indicates cdk 4 or cdk 2. The band to the right of each blot is the 2 h sense lysate immunoprecipitated and immunoblotted with cdk 2 or cdk 4 as a positive control. The thick band at the top of each blot is the heavy chain of IgG from the immunoprecipitation. The experiments shown are each representative of two separate experiments.

Figure 8 is a gel depicting PMA stimulated CKI induction is blunted in antisense $p21^{Waf1/Cip1}$ transfected cells, as described in Example II, *infra*. The experiments shown are representative of two separate experiments.

Figure 9 A-D are bar graphs showing Antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide has no significant effect DNA synthesis in PMA-inhibitable A431 cells, as described in Example II, *infra*. A431 or A10 cells were lipofected with from 0 to 400 nM of sense $p21^{Waf1/Cip1}$ or antisense $p21^{Waf1/Cip1}$. A431 cells were placed in serum-free medium overnight and then stimulated with (A) PDGF-BB (30 ng/ml), (B) 10% serum-containing medium, or (C) PDGF-BB or PMA (100 ng/ml) for another 8 h before [$^3$H]-thymidine was added for overnight incubation. (D) A10 VSM cells were transfected with sense and antisense $p21^{Waf1/Cip1}$ oligonucleotides as above and treated similarly to (B). DNA synthesis was assessed by [$^3$H]-thymidine incorporation and is expressed as mean ± s.e.m. of three wells per data point. The absolute counts differ between experiments due to different confluency of the cells. The experiments shown are representative of two separate experiments.

Figure 10 is a Western blot showing levels of p53 protein were not altered in A431 cells as compared to A10 VSM cells, as described in Example II, *infra*. The experiment shown is representative of two separate experiments.

Figure 11 is a Western blot showing Serum-induced hyperphosphorylation of Rb was not altered in A431 cells, as described in Example II, *infra*. The experiment shown is representative of two separate experiments.

Figure 12 is a gel showing Antisense $p21^{Waf1/Cip1}$ altered cyclin D1/cdk4 association in A431 cells, as described in Example II, *infra*. The arrowhead indicates cdk4 (top blot) or cdk2 (bottom blot). The band to the right of each blot is the 2 h sense lysate immunoprecipitated and immunoblotted with cdk2 or cdk4 as a positive control. The thick band at the top of each blot is the heavy chain of IgG from the immunoprecipitation. The experiments shown are each representative of two separate experiments.

Figure 13 is a bar graph showing antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide potentiates the cell cycle inhibitory (and presumably killing) effect of γ-irradiation on VSM cells

exposed to serum, as described in Example III *infra*. The experiment shown is representative of two separate experiments.

Figure 14 A and B illustrate how ionizing radiation inhibits DNA synthesis in VSM, but not A431, cells, as described in Example III, *infra*. Confluent (A) A10 VSM or (B) A431 cells were subjected to one of the following culture conditions: left in 10%-serum containing medium (continuous S); placed in serum-free medium the day of the experiment and left under those conditions for 48 h (continuous SF); placed in serum-free medium for 24 h and then stimulated with 10%-serum (SF→S) or PDGF-BB (30 ng/ml) (SF→PDGF-BB). All cells were irradiated with 8 Gy; when agonist was added, it was added 30 min after radiation. Six hours after agonist addition (where indicated), [$^3$H]-thymidine (1 μCi/ml) was added to the medium overnight and DNA synthesis was assessed. Data is expressed as mean ± s.e.m. of three wells per data point. * indicates $p<0.05$ compared to control (random sequence oligonucleotide). The experiments shown are representative of two separate experiments.

Figure 15 A and B show the induction of p21 in VSM cells by ionizing radiation is blunted by antisense oligonucleotide to p21, as described in Example III, *infra*. Confluent VSM cells were transfected with antisense oligonucleotide to p21 or random sequence control oligonucleotide as described in Materials and Methods. (A) Non-transfected cells were exposed to ionizing radiation (12 Gy), lysed at the indicated times after exposure, and Western blotted with p21 antibody. (B) Cells transfected with the indicated oligonucleotides were treated similarly to (A). The arrowhead shows the band corresponding to p21. The experiments shown are representative of two separate experiments.

Figure 16 depicts the induction of p21 in VSM cells by Adriamycin is blunted by antisense oligonucleotide to p21, as described in Example III, *infra*. The arrowhead shows the band corresponding to p21. The experiments shown are representative of two separate experiments.

Figure 17 illustrates how the antisense oligonucleotide to p21 potentiates radiation-induced VSM cell cycle arrest, as described in Example III, *infra*. The experiment shown is representative of two separate experiments. *,#$p<0.05$ compared to control; +$p<0.05$ compared to random sequence oligonucleotide.

Figure 18 demonstrates how the antisense oligonucleotide to p21 potentiates Adriamycin-induced VSM cell cycle arrest, as described in Example III, *infra*. The experiment shown is representative of two separate experiments. *,#$p<0.05$ compared to control; +$p<0.05$ compared to random sequence oligonucleotide.

Figure 19 A and B depict how Caspase-3 is activated by antisense oligonucleotide to p21 but not early after radiation or Adriamycin, as described in Example III, *infra*. Confluent VSM cells were transfected with oligonucleotide in the concentration indicated, left in serum-containing media overnight, and exposed to (A) ionizing radiation (12 Gy) or (B) Adriamycin where indicated. 4 h later, activation of caspase-3 was assessed by Western blotting. The arrowhead indicates the cleavage product of caspase-3 signifying its processing as an early step in apoptosis. Wortmannin (wort) is a positive control for apoptosis. The arrowhead shows the band corresponding to the cleavage product of caspase-3. The experiment shown is representative of two separate experiments.

Figure 20A-D show how the antisense oligonucleotide to p21 induces VSM cell apoptosis, as described in Example III, *infra*. VSM cells were grown on glass cover slips and transfected with (A,B) random sequence control oligonucleotide to p21 or (C,D) antisense oligonucleotide. 24 h later, the cells were fixed and stained *in situ* with Hoechst 33258. Representative microscopic fields were photographed under (A,C) visual or (B,D) UV light at 40x.

Figure 21 shows that TGF–β decreases mitogenesis in serum-starved VSM cells, as described in Example IV, *infra*. * indicates significance difference from control (no TGF–β). The experiment shown is representative of three separate experiments.

Figure 22 shows how TGF–β decreases 10% serum-stimulated mitogenesis in VSM cells cells, as described in Example IV, *infra*. * indicates significance difference from serum alone. The experiment shown is representative of two separate experiments.

Figure 23 demonstrates the transfection of VSM cells with antisense p21 oligodeoxynucleotide specifically inhibits p21 protein level cells, as described in Example IV, *infra*. The experiment shown is representative of at least three separate experiments.

Figure 24 illustrates that TGF–β remains inhibitory in VSM cells transfected with antisense p21 oligodeoxynucleotide cells, as described in Example IV, *infra*. A10 VSM cells were grown to confluence, transfected as described with either antisense (solid bars) or control (hatched bars) oligodeoxynucleotide, and serum-starved overnight. Subsequently, the cells were treated with 10% serum containing medium and/or TGF–β at the indicated concentrations (in ng/ml), and DNA synthesis assessed as in Figure 22; absolute counts differ slightly from other experiments due to differences in starting confluency of the cells. * indicates significance difference from serum alone. The experiment shown is representative of two separate experiments.

Figure 25 shows that the antisense p21 oligodeoxynucleotide decreases TGF–β-mediated laminin production and secretion cells, as described in Example IV, *infra*. The experiment shown is representative of three separate experiments.

Figure 26 demonstrates how the antisense p21 oligodeoxynucleotide decreases TGF–β-mediated fibronectin production and secretion cells, as described in Example IV, *infra*. The experiment shown is representative of three separate experiments.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising discovery that $p21^{Waf1/Cip1}$ protein serves a permissive role in PDGF-mediated cell growth and proliferation, such that its presence

is required for the mitogenic effect of this growth factor, for example, in VSM cells. Therefore, successful therapy and prevention of abnormal growth and proliferation of cells must take into account $p21^{Waf1/Cip1}$ activity or function to effectively combat proliferation of cells that result in disease states.

The methods and compositions of the invention can be used to treat a variety of diseases associated with abnormal cell growth and proliferation, including, but not limited to, atherosclerosis, angioplasty restenosis, renal mesangial cell proliferation and cancer, as well as preventing the VSM cell proliferation seen after coronary angioplasty, and may additionally be useful in cancer treatment as a sensitizer to chemotherapy and/or radiation (Mueller et al., 2000 *Cancer Res.* 2000. 60.(1):156.-63., 60, p. 156-163; and Wouters et al., 1997 *Cancer Res.*, 57, p. 4703-4706). The methods may also be used to prevent plaques or tumors from forming.

The methods of the invention include regulation of cell growth mediated by CDKs by inhibiting $p21^{Waf1/Cip1}$, using a $p21^{Waf1/Cip1}$ inhibitory agent to suppress abnormal cell growth and proliferation in VSM cells and other cells, including tumors (Mueller et al., 2000 *Cancer Res.*2000.Jan.1;60.(1):156.-63., 60, p. 156-163; and Wouters et al., 1997 *Cancer Res.*, 57, p. 4703-4706).

**Definitions**

As used herein a "$p21^{Waf1/Cip1}$" and "p21" are used interchangeably.

Inhibition of cell growth and proliferation, as used herein, means an effective decrease in the number of cells treated with the compound of the invention e.g. antisense oligonucleotide of p21, as compared to non-treated cells.

As used herein a "$p21^{Waf1/Cip1}$ inhibitory agent" is an agent that directly or indirectly inhibits activity of $p21^{Waf1/Cip1}$. A direct inhibitory agent, for example, is an antibody or antagonist that binds to and inhibits the activity of $p21^{Waf1/Cip1}$, soluble forms and

fragments thereof having $p21^{Waf1/Cip1}$-binding activity, and new $p21^{Waf1/Cip1}$ antagonists developed using well known methods for drug discovery as described herein and in the art. If the agent is $p21^{Waf1/Cip1}$ specific (i.e. a direct inhibitory agent), it prevents proliferation of cells at the site of abnormal proliferation, such as the heart or the vascular system. An indirect inhibitor, such as an antisense oligonucleotide of $p21^{Waf1/Cip1}$, inhibits the synthesis or secretion of $p21^{Waf1/Cip1}$, by binding to the nucleic acid sequence of $p21^{Waf1/Cip1}$ and/or inhibits the expression (i.e. transcription or translation) of $p21^{Waf1/Cip1}$, thereby reducing the amount of $p21^{Waf1/Cip1}$ produced, or sequestering it away from its target protein.

**Methods and Compositions of the Invention**

The present invention provides methods and compositions to treat diseases associated with abnormal cell proliferation, by inhibiting the expression or activity of $p21^{Waf1/Cip1}$. In one embodiment, a $p21^{Waf1/Cip1}$ inhibitory agent is administered to a subject at high risk for such diseases, for example atherosclerosis to prevent abnormal proliferation. Such high risk individuals can be prescreened using known medical procedures such as serum cholesterol measurements, history of premature heart disease, and invasive and non-invasive measurements of cardiac ischemia.

Included within the scope of $p21^{Waf1/Cip1}$ indirect inhibitors of the invention are nucleic acids, including antisense oligonucleotides, that block the expression of $p21^{Waf1/Cip1}$ genes within cells by binding a complementary messenger RNA (mRNA) and preventing its translation (Wagner, *Nature* 372:332-335 (1994); and Crooke and Lebleu, *Antisense Research and Applications*, CRC Press, Boca Raton (1993)). Gene inhibition may be measured by determining the degradation of the target RNA. Antisense DNA and RNA can be prepared by methods known in the art for synthesis of RNA including chemical synthesis such as solid phase phosphoramidite chemical synthesis or *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule.

The antisense DNA sequences may be incorporated into vectors with RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines. The potency of antisense oligonucleotides for inhibiting $p21^{Waf1/Cip1}$ may be enhanced using various methods including: 1) addition of polylysine (Leonetti et al., *Bioconj. Biochem.* 1:149-153 (1990)); 2) encapsulation into antibody targeted liposomes (Leonetti et al., *Proc. Natl. Acad. Sci. USA* 87:2448-2451 (1990) and Zelphati et al., *Antisense Research and Development* 3:323-338 (1993)); 3) nanoparticles (Rajaonarivony et al., *J. Pharmaceutical Sciences* 82:912-917 (1993) and Haensler and Szoka, *Bioconj. Chem.* 4:372-379 (1993)), 4) the use of cationic acid liposomes (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Capaccioli et al., *Biochem. Biophys. Res. Commun.* 197:818-825 (1993); Boutorine and Kostina, *Biochimie* 75:35-41 (1993); Zhu et al., *Science* 261:209-211 (1993); Bennett et al., *Molec. Pharmac.* 41:1023-1033 (1992) and Wagner, *Science* 280:1510-1513 (1993)); 5) Sendai virus derived liposomes (Compagnon et al., *Exper. Cell Res.* 200:333-338 (1992) and Morishita et al., *Proc. Natl. Acad. Sci. USA* 90:8474-8478 (1993)), to deliver the oligonucleotides into cells: (6) the conjugation of the antisense oligonucleotides to a fusogenic peptide, e.g. derived from an influenza hemagglutinin envelope protein (Bongartz et al., *Nucleic Acids Res.* 22(22):4681-4688 (1994)).

Also included within the scope of p21 direct inhibitors of the invention are antagonists which bind to p21. The term " antagonists," as it is used herein, refers to a molecule which, when bound to p21, decreases the amount or the duration of the effect of the biological activity of p21. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules which modulate the cell proliferation effects of p21. Suitable p21 antagonists can be readily determined using methods known in the art to screen candidate agent molecules for binding to p21, such as assays for detecting the ability of a candidate agent to measure CDK immunoprecipitation and check effect of immunopreciptated CDK on Rb phosphorylation (Sherr, C.J and Roberts J. M. *Genes and Development*, 13, 1501-1512 (1999)).

Direct inhibitors such as antibodies of the invention include polyclonal, monoclonal, chimeric, fragments, and humanized antibodies, that bind to p21 proteins or fragments of p21 proteins thereof. The most preferred antibodies will selectively bind to p21 proteins and will not bind (or will bind weakly) to non-p21 proteins. These antibodies can be from any source, e.g., rabbit, sheep, rat, dog, cat, pig, horse, mouse and human.

As will be understood by those skilled in the art, the regions or epitopes of a p21 protein to which an antibody is directed may vary with the intended application. For example, antibodies intended for use in an immunoassay for the detection of membrane-bound p21 on viable cells should be directed to an accessible epitope. The p21 proteins represents potential markers for screening, diagnosis, prognosis, and follow-up assays and imaging methods. In addition, based on the discoveries described herein, p21 proteins may be excellent targets for therapeutic methods such as targeted antibody therapy, immunotherapy, and gene therapy to treat conditions associated with the presence or absence of p21 proteins. Antibodies that recognize other epitopes may be useful for the identification of p21 within damaged or dying cells, for the detection of secreted p21 proteins or fragments thereof. Additionally, some of the antibodies of the invention may be internalizing antibodies, which internalize (e.g., enter) into the cell upon or after binding. Internalizing antibodies are useful for inhibiting cell growth and/or inducing cell death.

The invention includes a monoclonal antibody, the antigen-binding region of which competitively inhibits the immunospecific binding of any of the monoclonal antibodies of the invention to its target antigen. Further, the invention provides recombinant proteins comprising the antigen-binding region of any the anti-p21 monoclonal antibodies of the invention.

The invention also encompasses antibody fragments that specifically recognize a p21 protein or a fragment thereof. As used herein, an antibody fragment is defined as at least a portion of the variable region of the immunoglobulin molecule that binds to its target, i.e., the antigen binding region. Some of the constant region of the immunoglobulin may

be included. Fragments of the monoclonal antibodies or the polyclonal antisera include Fab, $F(ab')_2$, Fv fragments, single-chain antibodies, and fusion proteins which include the immunologically significant portion (i.e., a portion that recognizes and binds p21).

The chimeric antibodies of the invention are immunoglobulin molecules that comprise at least two antibody portions from different species, for example a human and non-human portion. Chimeric antibodies are useful, as they are less likely to be antigenic to a human subject than antibodies with non-human constant regions and variable regions. The antigen combining region (variable region) of a chimeric antibody can be derived from a non-human source (e.g. murine) and the constant region of the chimeric antibody, which confers biological effector function to the immunoglobulin, can be derived from a human source (Morrison et al., 1985 *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 ; Takeda et al., 1985 *Nature* 314:452; Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397). The chimeric antibody may have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule.

The chimeric antibodies of the present invention also comprise antibodies which are chimeric proteins, having several distinct antigen binding specificities (e.g. anti-TNP: Boulianne et al., 1984 *Nature* 312:643; and anti-tumor antigens: Sahagan et al., 1986 *J. Immunol.* 137:1066). The invention also provides chimeric proteins having different effector functions (Neuberger et al., 1984 *Nature* 312:604), immunoglobulin constant regions from another species and constant regions of another immunoglobulin chain (Sharon et al., 1984 *Nature* 309:364); Tan et al., 1985 *J. Immunol.* 135:3565-3567). Additional procedures for modifying antibody molecules and for producing chimeric antibody molecules using homologous recombination to target gene modification have been described (Fell et al., 1989 *Proc. Natl. Acad. Sci. USA* 86:8507-8511).

Humanized antibodies directed against p21 proteins are also useful. As used herein, a humanized p21 antibody is an immunoglobulin molecule which is capable of binding to a p21 protein. A humanized p21 antibody includes variable regions having substantially the

amino acid sequence of a human immunoglobulin and the hyper-variable region having substantially the amino acid sequence of non-human immunoglobulin. Humanized antibodies can be made according to several methods known in the art (Teng et al., 1983 *Proc. Natl. Acad. Sci. U.S.A.* 80:7308-7312; Kozbor et al., 1983 *Immunology Today* 4:7279; Olsson et al., 1982 *Meth. Enzymol.* 92:3-16).

Various methods for the preparation of antibodies are well known in the art. For example, antibodies may be prepared by immunizing a suitable mammalian host with an immunogen such as an isolated p21 protein, peptide, fragment, or an immunoconjugated form of p21 protein (Harlow 1989, in: *Antibodies*, Cold Spring Harbor Press, NY). In addition, fusion proteins of p21 may also be used as immunogens, such as a P21 fused to -GST-, -human Ig, or His-tagged fusion proteins. Cells expressing or overexpressing p21 proteins may also be used for immunizations. Similarly, any cell engineered to express p21 proteins may be used. This strategy may result in the production of monoclonal antibodies with enhanced capacities for recognizing endogenous p21 proteins (Harlow and Lane, 1988, in: *Antibodies*: A Laboratory Manual. Cold Spring Harbor Press).

The amino acid sequence of p21 proteins, and fragments thereof, may be used to select specific regions of the p21 proteins for generating antibodies. For example, hydrophobicity and hydrophilicity analyses of the p21 amino acid sequence may be used to identify hydrophilic regions in the p21 protein structure. Regions of the p21 protein that show immunogenic structure, as well as other regions and domains, can readily be identified using various other methods known in the art (Rost, B., and Sander, C. 1994 *Protein* 19:55-72), such as Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis. Fragments including these residues are particularly suited in generating anti-p21 antibodies.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. Techniques for conjugating or joining therapeutic agents to antibodies are well known (Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In

Cancer Therapy", in: *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in: *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in: *Monoclonal Antibodies '84: Biological And Clinical Applications,* Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58 (1982); Sodee et al., 1997, *Clin. Nuc. Med.* 21: 759-766). In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective.

Administration of a p21 immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein (*Nature* 256: 495-497) or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the p21 protein or a fragment thereof. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid. The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant.

The antibodies or fragments may also be produced by recombinant means. The antibody regions that bind specifically to the desired regions of the p21 protein can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin.

The antibodies of the invention bind specifically to polypeptides having p21 sequences. In one embodiment, the p21 antibodies specifically bind to the extracellular domain of a p21 protein. In other embodiments, the antibodies of the invention specifically bind to other domains of a p21 protein or precursor, for example the antibodies bind to the cytoplasmic domain of p21 proteins.

Additionally, some of the antibodies of the invention are internalizing antibodies, i.e., the antibodies are internalized into the cell upon or after binding (Liu, H. et al., *Cancer Res.* **1998**, 58, 4055-4060).

The methods of the invention comprise introducing the direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents so as to inhibit the activity of $p21^{Waf1/Cip1}$. For example, an anti-p21 mAb can be introduced into a subject to contact p21 positive cells to inhibit the activity of p21 and decrease the proliferation of cells.

In addition, the invention provides a process for the production of vaccines using p21 protein and a vaccine for treating cyclin-dependent kinase-mediated cell growth and proliferation. The vaccines contain a p21 protein, or partial sequences thereof, which is carrier-bound if desired, as an immunogen in a pharmacologically effective dose, and in a pharmaceutically acceptible formulation.

The production of these vaccines can be carried out according to known methods. However, the p21 proteins are preferably first lyophilized and subsequently suspended, if desired with addition of auxiliary substances.

Vaccination with these vaccines or combinations of vaccines according to the present invention can be carried out according to methods familiar to one skilled in the art (e.g. intradermally, intramuscularly, intraperitoneally, intravenously, subcutaneously or intranasally).

For intramuscular or subcutaneous administration, the vaccine can, for example, be suspended in physiological saline. For an intranasal or intraoccular application, the vaccine can, e.g., be used in the form of a spray or an aqueous solution. For a local, e.g. oral, administration, it is often necessary to temporarily protect the immunogens against inactivation, for example against proteolytic enzymes in the cavity of the mouth or in the stomach. Such temporary protection can be achieved by encapsulating the immunogens. This encapsulation can be carried out by coating with a protective agent (microencapsulation) or by embedding a multitude of immunogens according to the present invention in a protective carrier (macroencapsulation).

The encapsulation material can be semipermeable or become semipermeable when introduced into the human or animal body. A biologically degradable substance is usually used as a carrier for the encapsulation.

**Administration of Inhibitors**

The direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents may be administered to mammalian subjects, including: humans, monkeys, apes, dogs, cats, cows, horses, rabbits, mice and rats. The methods include administration by standard parenteral routes, such as subcutaneously, intravenously, intramuscularly, intracutaneously, intra-articularly, intrasynovially, intrathecally, periostally, or by oral routes. Alternative methods include, administration by implantable pump or continuous infusion, injection, or liposomes. Administration can be performed daily, weekly, monthly, every other month, quarterly or any other schedule of administration as a single dose injection or infusion, multiple dose, or in continuous dose form.

As is standard practice in the art, the direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents of the invention may be administered to the subject in any pharmaceutically acceptable carrier or adjuvant which is known to those of skill of the art. These carriers and adjuvants include, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid,

potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances and polyethylene glycol.

The direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents may be administered to a subject in an amount and for a time sufficient to block the activity of $p21^{Waf1/Cip1}$, in the subject. The amount and time may also be sufficient to block $p21^{Waf1/Cip1}$ positive cells direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents. The most effective mode of administration and dosage regimen for the inhibitors in the methods of the present invention depend on the severity of the abnormal proliferation of cells, the subject's health, previous medical history, age, weight, height, sex, response to treatment and the judgment of the treating physician. Therefore, the amount of inhibitors to be administered, as well as the number and timing of subsequent administrations are to be determined by a medical professional conducting therapy based on the response of the individual subject. Initially, such parameters are readily determined by skilled practitioners using appropriate testing in animal models for safety and efficacy, and in human subjects during clinical trials of candidate therapeutic inhibitor formulations. To determine if the amount administered is sufficient, the subject may be monitored for certain symptoms associated with the abnormal proliferation of cells.

Disruption of p53 (Bunz F, et al. *J Clin Invest* 104:263-269, 1999), and also of p21 (Wouters BG, et al. *Cancer Res* 57:4703-4706, 1997), sensitizes cancer cells to DNA damaging agents. Therefore, using the inhibitors of the invention in the methods of the invention, vascular cells may be rendered more sensitive to the effects of DNA damaging agents, such that targeted cells or tissues may be made more likely to become growth arrested and subsequently apoptotic, after p21 levels are attenuated. The invention also encompasses the use of the direct or indirect $p21^{Waf1/Cip1}$ inhibitory agents of the invention together with other chemotherapeutic agents, such as adriamycin cisplatinum, carboplatin, vinblastine, vincristine, taxol , dactinomycin (actinomycin D), daunorubicin (daunomycin, rubidomycin), bleomycin, plicamycin (mithramycin), mitomycin

(mitomycin C), methotrexate, cytarabine (AraC), azauridine, azaribine, fluorodeoxyuridine, deoxycoformycin, and mercaptopurine. In addition, those of skill in the art will appreciate that the compounds of the present invention can be used in conjunctive therapy with other known chemotherapeutic compounds.

The Examples, *infra*, include the demonstration that transfection of several lines of VSM cells with antisense oligodeoxynucleotide specific to $p21^{Waf1/Cip1}$ correlated with decreased cyclin D1/cdk 4, but not cyclin E/cdk 2 association. The Examples also show a dose-dependent inhibition of PDGF-BB-stimulated DNA synthesis and cell proliferation. The Examples demonstrate that the presence of $p21^{Waf1/Cip1}$ is required for growth factor-induced proliferation of VSM cells.

The following examples are presented to demonstrate the methods of the present invention and to assist one of ordinary skill in using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure of the protection granted by Letters Patent granted hereon.

## EXAMPLE I

### $p21^{Waf1/Cip1}$ Is Required For PDGF Induced Vascular Smooth Muscle Cell Proliferation

**Materials**: Human recombinant PDGF-BB was obtained from Upstate Biotechnology, Inc (UBI)(Lake Placid, NY). Mouse monoclonal $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ and cyclin D1, goat polyclonal cdk 2 and cdk 4, and rabbit polyclonal cyclin E antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat horseradish peroxidase-conjugated IgG was obtained from BioRad (Richmond, CA). Lipofectin® was obtained form Life Technologies (Rockville, MD). Reagents for the Enhanced Chemiluminescence system and [$^3$H]thymidine were obtained from Amersham (Arlington Heights, IL). All other reagents, including mouse monoclonal α-actin antibody, were from Sigma (St. Louis, MO).

**Cell culture, DNA synthesis, and proliferation assays:** Cultures of both A10 and A7r5 rat aortic VSM cells were obtained from American Type Culture Collection (Rockville MD). Bovine aortic smooth muscle cells were supplied by Martha O'Donnell (O'Donnell and Owen, 1986 *Proc .Natl. Acad. Sci.U.S.A.*, 83, p. 6132-6136). All of the cell lines were maintained as described (Weiss et al., 1998 *Am. J. Physiol.*, 274, p. C1521-C1529) and were used between passages 15 and 25. The cells were growth-arrested by placing them in serum-free quiescence medium, exposed to growth factors as indicated, and [$^3$H]thymidine incorporation assessed as previously described (Weiss and Nuccitelli, 1992a *J. Biol. Chem.*, 267, p. 5608-5613). Cell proliferation was assessed by counting of adherent cells on 4 representative fields under 100x magnification in each of 3 wells per experimental condition.

**Antisense transfections:** Phosphorothioate antisense oligodeoxynucleotides were synthesized by Oligonucleotides Etc. (Wilsonville, OR). The $p21^{Waf1/Cip1}$ antisense vector was designed around the start codon of rat $p21^{Waf1/Cip1}$, with sequence 5'-GAC ATC ACC AGG ATC GGA CAT-3' (SEQ. ID NO.:1). The sense $p21^{Waf1/Cip1}$ sequence is 5'-ATG TCC GAT CCT GGT GAT GTC-3' (SEQ. ID NO.:2). The scrambled random sequence control oligodeoxynucleotide was 5'-TGG ATC CGA CAT GTC AGA-3' (SEQ. ID NO.:3). For the lipofection procedure, cells were grown to 90% confluence, the appropriate concentration of oligodeoxynucleotide was mixed with 6.6 μL of Lipofectin® per ml of Opti-MEM medium and was added to the cells for 4 h at 37°C. The cells were washed and serum-free medium (without oligodeoxynucleotide) was added overnight, the media was changed in the morning and the cells were incubated in serum-free medium for the times indicated.

**Western blots:** Cells were grown to confluence in 6 cm culture dishes and serum deprived. After transfection and or treatment with appropriate agonist, the cells were washed with phosphate-buffered saline and lysed in lysis buffer and the supernatant was Western blotted as described (Weiss et al., 1998 *Am. J. Physiol.*, 274, p. C1521-C1529).

1. Determination of the Dependence of G1-phase progression on $p21^{Waf1/Cip1}$ in the VSM cell lines.

Antisense techniques were employed to examine the dependence of G1-phase progression on $p21^{Waf1/Cip1}$ in the VSM cell lines (Crooke, 1993 *Antisense research and applications*.. Boca Raton, CRC.). The oligodeoxynucleotides used were generated around the ATG start codon using GenBank sequences and were screened for lack of stable secondary structures or stable homodimer formation (OligoTech software, Oligonucleotides Etc., Wilsonville, OR). Three independent controls were used in these experiments: (i) "dummy" transfection with Lipofectin but no DNA, (ii) random sequence oligodeoxynucleotide (SEQ. ID NO.:3) transfection, and (iii) sense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:2) transfection. VSM cells were transfected with the appropriate oligodeoxynucleotide or control overnight in serum-free medium, and the next day the cells were stimulated with PDGF-BB (30 ng/ml) for another 18 h. DNA synthesis was assessed by [$^3$H]-thymidine incorporation and is expressed as mean ± s.e.m. of three wells per data point. The absolute counts differ between experiments due to different confluency of the cells. Significant inhibition of PDGF-stimulated DNA synthesis occurred when the cells were transfected with antisense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:1), but not with sense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:2), "dummy" transfection (Figure 1a), or random sequence (SEQ. ID NO.:3) control (Figure 1b). To confirm that the observed growth inhibition was specific to the antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1), dose/response analysis were performed. There was inhibition of DNA synthesis with increasing concentration of antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) up to 200 nM, with no effect of sense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:2) (Figure 1c). To demonstrate that this effect was not specific to the A10 cell line, a similar effect in a bovine VSM cell line (Figure 1d). A10 VSM cells were transfected as in Figure 1c. After 18 h of PDGF incubation, the cells were counted by examining representative fields at 100x magnification. The average number of cells in 4 random fields in each well was determined. Changes in cell number were shown to parallel the alterations in DNA synthesis (Figure 2).

2. Determination of Transfection Efficiency of the $p21^{Waf1/Cip1}$ Antisense Oligonucleotide Agents.

To establish whether the oligodeoxynucleotides crossed the cell membrane and entered the nucleus in order to inhibit $p21^{Waf1/Cip1}$ protein production, cells were transfected with a fluorescein-tagged $p21^{Waf1/Cip1}$ antisense oligodeoxynucleotide (with the same sequence as the $p21^{Waf1/Cip1}$ antisense) and were examined for transfection efficiency. Upon examination by fluorescence microscopy, these cells demonstrated 100% transfection efficiency (Figure 3), as has been reported for this technique (Coats et al., 1996 *Science*, 272, p. 877-880).3. Determination of Effect of $p21^{Waf1/Cip1}$ Antisense oligonucleotides on $p21^{Waf1/Cip1}$ Expression Levels.

To determine whether antisense transfection with $p21^{Waf1/Cip1}$ antisense oligonucleotides indeed decreases $p21^{Waf1/Cip1}$ protein levels, $p21^{Waf1/Cip1}$ levels after antisense transfection were examined employing the fact that PMA is a potent inducer of $p21^{Waf1/Cip1}$ (Huang et al., 1995 *Proc.Natl.Acad.Sci.U.S.A.*, 92, p. 4793-4797;Michieli et al., 1994 *Cancer Res.*, 54, p. 3391-3395). Since $p21^{Waf1/Cip1}$ protein levels were induced in VSM cells between 2 and 6 h after PMA stimulation (Figure 4), the $p21^{Waf1/Cip1}$ levels in transfected cells after similar times of PMA stimulation were examined. After transfection with appropriate oligodeoxynucleotide and subsequent overnight incubation in quiescent media, antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) caused significant attenuation of PMA-induced $p21^{Waf1/Cip1}$ levels in VSM cells up to 6 h (Figure 5). There was no effect of the $p21^{Waf1/Cip1}$ sense (SEQ. ID NO.:2) control oligodeoxynucleotide on cellular $p21^{Waf1/Cip1}$ levels (compare with Figure 4).

4. Determination of Specificity of $p21^{Waf1/Cip1}$ Antisense Oligonucleotides (SEQ. ID NO.:1).

To check for specificity of protein inhibition by the antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1), protein levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ after transfection with antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) were

examined. In these experiments the ability of antisense oligodeoxynucleotides (SEQ. ID NO.:1) to inhibit maximally stimulated CKI expression (see Figure 4) was assessed. The cells were stimulated with PMA for 4 h at various times after overnight serum starvation.

While antisense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:1) completely inhibited $p21^{Waf1/Cip1}$ protein even after maximal stimulation with PMA, there was a slight decrease in $p27^{Kip1}$ protein as well with this oligodeoxynucleotide (Figure 6a). At the times indicated after the overnight incubation (in hours), the medium was changed to quiescence medium and the cells were stimulated with PMA for 4 h to show maximal $p21^{Waf1/Cip1}$ expression (Figure 6). The lysates were immunoblotted with (a) $p21^{Waf1/Cip1}$ or $p27^{Kip1}$ antibody or (b) α-actin antibody. This is likely due to sequence similarity between the two genes, as $p21^{Waf1/Cip1}$ shares 43% sequence identity with $p27^{Kip1}$ in the cdk/cyclin binding site (residues 27-88), located in the conserved N terminus (Nomura et al., 1997 *Gene*, 191, p. 211-218;Toyoshima and Hunter, 1994 *Cell*, 78, p. 67-74).

Levels of the VSM cell structural protein α-actin were not altered after transfection under identical conditions (Figure 6b), demonstrating that the effect of antisense oligonucleotides (SEQ. ID NO.: 1) on cell proteins was not a general inhibitory one. Furthermore, it is not believed that the slight $p27^{Kip1}$ inhibition is playing a significant role in mitogenic inhibition, in light of data from other investigators (Rivard et al., 1996 *J.Biol.Chem.*, 271, p. 18337-18341) based on the cyclin/cdk data discussed below.

5. Determination of the Effect of $p21^{Waf1/Cip1}$ on the Association of Cyclin D1/cdk 4.

Cyclin D1/cdk 4 interaction was examined to determine this association as a possible mechanism of the permissive effect on growth of $p21^{Waf1/Cip1}$ in VSM cells. Because other CKIs, such as $p27^{Kip1}$, have been shown to affect cyclin E/cdk 2 interaction (Cheng et al., 1998 *Proc.Natl.Acad.Sci.U.S.A.*, 95, p. 1091-1096;Polyak et al., 1994 *Cell*, 78, p. 59-66), the nature of this association was also examined. While the CKIs have been shown to be growth inhibitors in VSM cells (Chang et al., 1995 *J. Clin. Invest.*, 96, p. 2260-2268; Fukui et al., 1997 *Atherosclerosis*, 132, p. 53-59; and Matsushita et al., 1998

*Hypertension*, 31, p. 493-498), various CKIs have been reported to act as "assembly factors" in other cells, both *in vivo* (Cheng et al., 1999 *EMBO J.*, 18, p. 1571-1583) and *in vitro* (LaBaer et al., 1997 *Genes Dev.*, 11, p. 847-862), yet previous studies have not shown inhibition of growth with interference of cyclin/cdk association. Since the cyclin D1/cdk 4 interaction occurs early after growth factor stimulation (reviewed in (Arellano and Moreno, 1997 *Int. J. Biochem. Cell Biol.*, 29, p. 559-573)) and because this interaction is facilitated by $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ *in vivo* (LaBaer et al., 1997: *Genes Dev.*, 11, p. 847-862).

Cells were transfected with $p21^{Waf1/Cip1}$ antisense (SEQ. ID NO.:1) or sense oligodeoxynucleotide (SEQ. ID NO.:2), allowed to grow overnight in serum-free media. After overnight incubation in serum-free medium, the cells were stimulated with PDGF-BB (30 ng/ml) for the times indicated. The cells were subsequently immunoprecipitated with either cyclin D1 or cyclin E and immunoblotted with cdk 4 or cdk 2, respectively. Antisense $p21^{Waf1/Cip1}$-transfected cells showed a marked decrease in association of cyclin D1 and cdk 4 at all times of PDGF stimulation, with no change in the cyclin E/cdk 2 interaction (Figure 7). Thus the inhibitory effect of antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) in VSM cells is likely by means of disruption in cyclin D1/cdk 4 interaction and thus prevention of activation of cdk 4 by cyclin D1.

Abnormal proliferation of VSM-like cells is pathogenic for a variety of diseases, such as atherosclerosis and angioplasty restenosis (Ross, 1993 *Nature*, 362, p. 801-809), as well as renal mesangial cell proliferation (Megyesi et al., 1999 *Proc. Natl. Acad. Sci.U.S.A.*, 96, p. 10830-10835), thus the mechanism by which these cells are stimulated to grow is important in designing antiproliferative therapies for treating these and other diseases. Published studies in VSM cells focus on the antiproliferative action of CKI overexpression (Chang et al., 1995 *J. Clin. Invest.*, 96, p. 2260-2268; Fukui et al., 1997 *Atherosclerosis*, 132, p. 53-59; Matsushita et al., 1998 *Hypertension*, 31, p. 493-498; and Smith et al., 1997: *Genes Dev.*, 11, p. 1674-1689), and there are even some studies promoting the idea that pharmacological methods to increase $p21^{Waf1/Cip1}$ may be useful in preventing the VSM cell proliferation seen after coronary angioplasty (Kusama et al.,

1999 *Atherosclerosis*, 143, p. 307-313; Takahashi et al., 1999 *Circ.Res.*, 84, p. 543-550; and Yang et al., 1996 *Semin. Interv. Cardiol.*, 1, p. 181-184).

The above results show for the first time that inhibition of $p21^{Waf1/Cip1}$ efficiently blocks mitogen stimulated VSM cell proliferation.

The difference between data presented herein, as compared to that in the mouse cells, may well be due to cell type, but the finding of growth inhibition in cells lacking active $p21^{Waf1/Cip1}$, explains the "essential activator" role of p21 promulgated by that group (Cheng et al., 1999 *EMBO J.*, 18, p. 1571-1583). Furthermore, since p21(-/-) mice appear to develop normally (Deng et al., 1995: *Cell*, 82, p. 675-684), it is conceivable that $p21^{Waf1/Cip1}$ disruption only affects "adult" cells, or that redundant pathways for cell growth are not present in A10 cells. Nevertheless, the data herein is the first demonstrating that the presence of these pleiotropic molecules is *required* for growth-factor mediated G1 progression in any cell type.

While the antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) clearly inhibits p21, there is also slight inhibition of $p27^{Kip1}$ protein as well by this oligodeoxynucleotide (Figure 3). This occurrence is likely due to the sequence similarity between the two genes (Nomura et al., 1997 *Gene*, 191, p. 211-218; and Toyoshima and Hunter, 1994 *Cell*, 78, p. 67-74). However, it is not believed that the slight amount of inhibition of $p27^{Kip1}$ is actively inhibiting VSM cell growth in the experiments, since cyclinE/cdk2 association is not affected (Coats et al., 1996 *Science*, 272, p. 877-880; Polyak et al., 1994 *Cell*, 78, p. 59-66; and Ravitz et al., 1995 *Cancer Res.*, 55, p. 1413-1416) (see Figure 7). Furthermore, others have reported suppression of quiescence, not of G1-phase progression, in fibroblasts transfected with antisense $p27^{Kip1}$ (Rivard et al., 1996: *J. Biol. Chem.*, 271, p. 18337-18341). In any case, the finding of unchanged α–actin protein levels in antisense p21 transfected cells argues against a general suppressive effect of these oligodeoxynucleotides (or of the process of transfection) on protein transcription.

Very recent work has shown that lack of a functional $p21^{Waf1/Cip1}$ gene in transgenic mice ameliorates progression of chronic renal failure after partial renal ablation (Megyesi et al., 1999 *Proc. Natl. Acad. Sci. U.S.A.*, 96, p. 10830-10835). PCNA was found to be significantly increased in p21(-/-) animals, but the degree of mesangial expansion was not quantitated. While the decrease in progression of renal failure was assumed to be due to a more hyperplastic (rather than hypertrophic) reaction in the p21(-/-) animals, the data disclosed in this example may shed some light on this phenomenon by suggesting that the response in p21(-/-) animals may have been a result of decreased mesangial cell mitogenesis due to inhibition of $p21^{Waf1/Cip1}$ expression. This point of view is reinforced by others, noting that "all kidney growth parameters reported by Megyesi et al (Megyesi et al., 1999 *Proc. Natl. Acad. Sci.U.S.A.*, 96, p. 10830-10835) are lower in p21(-/-) mice compared to p21(+/+) mice" (Al-Awqati and Preisig, 1999 *Proc. Natl. Acad. Sci.U.S.A.*, 96, p. 10551-10553).

Thus, these results support use of antisense $p21^{Waf1/Cip1}$ oligonucleotides (SEQ. ID NO.:1) to treat diseases involving abnormal VSM (or similar type) cell proliferation, such as atherosclerosis, angioplasty re-stenosis, and renal disease. While the available research on the CKIs in VSM cells has focused on the induction of the Cip/Kip family of CKIs in the presence of antiproliferative situations (Chen and Gardner, 1998 *J. Clin. Invest.*, 102, p. 653-662; Fukui et al., 1997 *Atherosclerosis*, 132, p. 53-59; Kusama et al., 1999 *Atherosclerosis*, 143, p. 307-313; and Perlman et al., 1998 *J. Biol. Chem.*, 273, p. 13713-13718), the inhibitory effects of CKI antisense constructs that were shown here may well be specific to vascular-like cells, as they were not observed in A431 cells (Example II, infra and reference (Ohtsubo et al., 1998 *Oncogene*, 16, p. 797-802).

## EXAMPLE II

**The Permissive Effect of $p21^{Waf1/Cip1}$ on DNA Synthesis in p53-inactive cells**

**Materials**: Human recombinant PDGF-BB was obtained from UBI (Lake Placid, NY). Mouse monoclonal $p21^{Waf1/Cip1}$ and cyclinD1, goat polyclonal cdk2 and cdk4, and rabbit

polyclonal cyclinE antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat horseradish peroxidase-conjugated IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectin® was obtained form Life Technologies (Rockville, MD). Reagents for the Enhanced Chemiluminescence system and [$^3$H]thymidine were obtained from Amersham (Arlington Heights, IL). All other reagents, including mouse monoclonal α–actin antibody, were from Sigma (St. Louis, MO).

**Cell culture and DNA synthesis**: Cultures of A10 and A431 cells were obtained from American Type Culture Collection (Rockville MD), were maintained as described (Weiss et al. 1998, *Am. J. Physiol.* 274, C1521-C1529), and were used between passages 15 and 24 or 25 and 35, respectively. The cells were growth-arrested by placing them in serum-free quiescence medium, exposed to growth factors as indicated, and [$^3$H]thymidine incorporation assessed as previously described (Weiss and Nuccitelli, 1992, *J. Biol. Chem.* 267, 5608-5613).

**Antisense transfections**: Phosphorothioate antisense oligodeoxynucleotides were synthesized by Oligonucleotides Etc. (Wilsonville, OR). The $p21^{Waf1/Cip1}$ antisense vector was designed around the start codon of rat $p21^{Waf1/Cip1}$, with sequence 5'-GAC ATC ACC AGG ATC GGA CAT-3' (SEQ. ID NO.:1). The sense $p21^{Waf1/Cip1}$ sequence is 5'-ATG TCC GAT CCT GGT GAT GTC-3' (SEQ. ID NO.:2). The scrambled random sequence control oligodeoxynucleotide was 5'-TGG ATC CGA CAT GTC AGA-3' (SEQ. ID NO.:3). For the lipofection procedure, cells were grown to 90% confluence, the appropriate concentration of oligodeoxynucleotide was mixed with 6.6 μL of Lipofectin® per ml of Opti-MEM medium and was added to the cells for 4 h at 37°C. Serum-free medium (without oligodeoxynucleotide) was added overnight, the media was changed in the morning and the cells were incubated in serum-free medium for the times indicated.

**Western blots**: Cells were grown to confluence in 6 cm culture dishes and serum deprived. After transfection and or treatment with appropriate agonist, the cells were washed with phosphate-buffered saline and lysed in lysis buffer and the supernatant was

Western blotted as described (Weiss and Nuccitelli, 1992 *J. Biol. Chem.*, 267, p. 5608-5613).

Example I shows that the CKI $p21^{Waf1/Cip1}$, while growth inhibitory in most situations, can also serve a permissive role in VSM cell growth. The mechanism behind this biphasic phenomenon is not yet known. In an attempt to further elucidate the nature of this effect, this study was designed to examine a cell line which is deficient in the immediate upstream regulator of $p21^{Waf1/Cip1}$. A431 cells, derived from a human squamous carcinoma, possess an inactive p53 protein (Kwok et al., 1994 *Cancer Res.*, 54, p. 2834-2836) and are thus useful for assessing p53-independent effects of $p21^{Waf1/Cip1}$.

1. Determination of the Effect of $p21^{Waf1/Cip1}$ in the A431 Cells.

In order to determine whether $p21^{Waf1/Cip1}$ exerts a permissive effect on growth in cells which do not possess an active p53 protein, oligodeoxynucleotides encoding antisense (SEQ. ID NO.:1) and sense (SEQ. ID NO.:2) sequences which were generated around the ATG translational start codon were utilized as in Example I.

PMA is a potent inducer of $p21^{Waf1/Cip1}$ in a variety of different cell lines (Huang et al., 1995 *Proc.Natl.Acad.Sci.U.S.A.*, 92, p. 4793-4797;Michieli et al., 1994 *Cancer Res.*, 54, p. 3391-3395) and was employed to examine $p21^{Waf1/Cip1}$ levels after antisense transfection and confirm efficacy of translation to attenuate $p21^{Waf1/Cip1}$ levels. A431 cells were transfected with 200 nM antisense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:1) or sense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:2). After overnight incubation in serum-free medium, the cells were stimulated with PMA (100 ng/ml) for the times indicated, lysed, and the lysates were Western blotted with $p21^{Waf1/Cip1}$ or α-actin antibody. Antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) caused significant attenuation of PMA-induced $p21^{Waf1/Cip1}$ levels in A431 cells up to 6 h; there was no effect of the $p21^{Waf1/Cip1}$ sense control oligodeoxynucleotide (SEQ. ID NO.:2) on cellular $p21^{Waf1/Cip1}$ levels as compared to other p53-independent cell lines (Zeng and el-Deiry, 1996, *Oncogene* 12, 1557-1564) when stimulated with phorbol ester (Figure 8). Levels

of the structural protein α-actin were not altered after transfection under identical conditions (Figure 8), demonstrating that the effect of antisense oligonucleotides (SEQ. ID NO.:1) on cell proteins was not a general inhibitory one towards protein translation.

2. Effect of $p21^{Waf1/Cip1}$ as an "Assembly Factor" Role in Growth of A431 Cells

In order to determine whether $p21^{Waf1/Cip1}$ serves an "assembly factor" role in growth of A431 cells as was observed in VSM cells of Example I, the DNA synthesis in these cells after transfection with antisense (SEQ. ID NO.:1) or sense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:2) oligodeoxynucleotides and subsequent stimulation with PDGF or serum was examined. There was no significant change in DNA synthesis upon stimulation of the cells with both of these growth factors (Figure 9a,b), suggesting that $p21^{Waf1/Cip1}$ does not serve an essential role in growth in A431 cells. Further experiments showed no difference in DNA synthesis between sense (SEQ. ID NO.:2) and antisense oligodeoxynucleotide (SEQ. ID NO.:1) up to 800 nM (data not shown). As a control for the ability of the transformed A431 cell line to in fact be growth inhibited, these cells were incubated with PMA and showed significant growth inhibition with this agent (Figure 9c) as has been demonstrated in variety of other cell lines (Weiss et al., 1991 *J. Cell. Physiol.*, 149, p. 307-312; and Weiss and Yabes, 1996 *Am. J. Physiol.(Cell Physiol.)*, 270, p. C619-C627). This A431 data is in contrast with that obtained in a variety of VSM cell lines, where cell growth was inhibited by similar oligodeoxynucleotide concentrations in cell stimulated with both PDGF-BB and serum (Figure 9d).

3. Determination of Mechanism of A431 vs VSM Cells Affected by Antisense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:1).

To begin to examine the mechanism by which A431 carcinoma cells are disparately affected by antisense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:1) as compared to VSM cells, the Rb status of these cells compared with A10 VSM cells was first examined. It is known that A431 cells possess a mutant p53 protein which renders this protein inactive (Kwok et al., 1994 *Cancer Res.*, 54, p. 2834-2836). Lysates from non-serum-starved A431 and A10

cells were Western blotted with p53 antibody which recognizes both wild type and mutant forms of p53. DNA damaging agents are able to up-regulate the mutant form of this protein in these cells (Kwok et al., 1994 *Cancer Res.*, 54, p. 2834-2836), yet, despite the differences in p53 activity in the two cell types, similar levels of this protein are found in both lines (Figure 10).

Upon activation by cyclin/cdk complexes, the Rb protein in turn becomes phosphorylated, causing it to release the transcription factors known as E2F, leading to early oncogene expression and ultimately to cell growth. An effect of $p21^{Waf1/Cip1}$ on cdk/Rb interaction would be evident by a change in phosphorylation state of the Rb protein: this property can be assessed by examining small changes in gel mobility of this protein. A431 cells were transfected with 400 nM antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) or with lipofectin only (no DNA). After overnight incubation in serum-free medium, the cells were stimulated with complete media for 6 or 24 h, lysed, and the lysates were Western blotted with Rb antibody. Rb became hyperphosphorylated after 6 and 24 h of 10% serum stimulation as evidenced by the appearance of a higher molecular weight band, and there was no difference between cells transfected (as control) with no DNA and those transfected with antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1) (Figure 11), suggesting that $p21^{Waf1/Cip1}$ has a minimal, if any, role in regulating this very distal part of the mitogenic signaling pathway in p53-inactive A431 cells.

Cyclin/cdk interactions occur after growth factor stimulation and serve to integrate such responses with the CKIs and transmit them to the Rb/E2F systems, which leads to mitogenic signal transmission. The cyclin D1/cdk 4 interaction occurs early after growth factor stimulation (reviewed in (Arellano and Moreno, 1997 *Int. J. Biochem. Cell Biol.*, 29, p. 559-573)), while the cyclinE/cdk2 interaction occurs late in G1 and is thought to have a role in triggering the actual onset of DNA replication after the cells have passed the restriction point (reviewed in (Sherr and Roberts, 1995: *Genes Dev.*, 9, p. 1149-1163)). A431 cells were transfected with $p21^{Waf1/Cip1}$ antisense (SEQ. ID NO.:1) or sense (SEQ. ID NO.:2) oligodeoxynucleotide, allowed to grow overnight in serum-free media,

and then stimulated for various times with PDGF-BB. The cells were subsequently immunoprecipitated with either cyclinD1 or cyclinE and immunoblotted with cdk4 or cdk2, respectively. A lysate sample, showing the mobility of the immunoprecipitated cdks, confirmed the identity of the cdks. A431 cells were transfected with antisense (SEQ. ID NO.:1) or sense $p21^{Waf1/Cip1}$ (SEQ. ID NO.:2)oligonucleotides. After overnight incubation in serum-free medium, the cells were stimulated with PDGF-BB (30 ng/ml) for the times indicated and immunoprecipitated (IP) with cyclin D1 or cyclin E and immunoblotted (IB) with cdk4 or cdk2. Unlike the situation in A10 VSM cells of Example I, there was no change in cyclinD/cdk4 association when $p21^{Waf1/Cip1}$ expression was inhibited by antisense oligodeoxynucleotide (SEQ. ID NO.:1) (Figure 12), suggesting that this protein is not required for association of these signaling proteins in these p53 inactive cells. Furthermore, there was no association of cyclinE with cdk2 at times ranging from 10 min to 6 h of PDGF exposure, and there was no effect of $p21^{Waf1/Cip1}$ inhibition in these experiments (Figure 12).

As shown above in Example I, that the CKI $p21^{Waf1/Cip1}$ can play a permissive role in growth of VSM cells, acting by allowing assembly of the cyclinD1/cdk4 complex, which in turn leads to events resulting in cell cycle transit. In Example II, the focus was on the direct upstream influence on $p21^{Waf1/Cip1}$: the tumor suppressor p53. To determine if p53 has any influence on the nature of the $p21^{Waf1/Cip1}$ effect (stimulatory versus inhibitory on cell growth) has not been examined.

Example II shows that the permissive effect of $p21^{Waf1/Cip1}$ on cell growth is not universal, as it does not occur in A431 cells stimulated either with PDGF-BB or serum. While the cell lines used, A431 and A10, are two distinct cell types, one a squamous carcinoma line and the other a smooth muscle line, the growth factor-stimulated mitogenic signaling pathways are believed to be quite similar, with the most obvious difference being that the A431 cells lack a functional p53 protein because of a mutation in the gene encoding this protein (Kwok et al., 1994 *Cancer Res.*, 54, p. 2834-2836). So the cells that display permissive effect of $p21^{Waf1/Cip1}$ on cell growth are the optimal targets of the present invention.

While the most likely explanation of this data is that a functional p53 protein is necessary for the permissive effect of $p21^{Waf1/Cip1}$, there exist several alternative scenarios. While it was observed that $p21^{Waf1/Cip1}$ protein expression is specifically decreased by the antisense $p21^{Waf1/Cip1}$ oligodeoxynucleotide (SEQ. ID NO.:1), it is conceivable that the magnitude of the decrement in A431 cells using antisense oligodeoxynucleotides was less than that seen with the VSM cells, or that A431 cells can signal to Rb with lower levels of $p21^{Waf1/Cip1}$ protein.

While not wishing to be bound by any theory, since it has been well established (El-Deiry et al., 1993, *Cell* 75, 817-825) that p53 induces transcription of the $p21^{Waf1/Cip1}$ gene (which would lead to an increase in $p21^{Waf1/Cip1}$ protein levels), it is possible that there exist extremely low levels of $p21^{Waf1/Cip1}$ in the absence of functional p53, as would occur in A431 cells. This may, in turn, result in the activation of alternative pathways which the cell has evolved to allow growth and circumvent any requirement for $p21^{Waf1/Cip1}$ in cell cycle transit. That there exist alternate pathways for p53-mediated cell cycle arrest independent of $p21^{Waf1/Cip1}$ is clear, since fibroblasts homozygous null for $p21^{Waf1/Cip1}$ are only partially defective in their response to DNA damage (Brugarolas et al., 1995 *Nature*, 377, p. 552-557;Deng et al., 1995 *Cell*, 82, p. 675-684). This possible mechanism is further supported by the finding that primary fibroblasts from $p21^{Waf1/Cip1}$ and $p27^{Kip1}$-null mice did not show overtly abnormal cell cycles, despite the finding by those investigators that overall cyclinD-dependent kinase activity was reduced below the assay limit of detectability (Cheng et al., 1999 *EMBO J.*, 18, p. 1571-1583). Still other studies have shown an increased growth rate of p21(-/-) as compared to wild type mouse embryonic fibroblasts (Deng et al., 1995 *Cell*, 82, p. 675-684), and no apparent G1 block in human colorectal cancer cells (Waldman et al., 1995 *Cancer Res.*, 55, p. 5187-5190). This is the first demonstration of the lack of a permissive role of $p21^{Waf1/Cip1}$ in cells deficient in active p53.

Cells lacking p53 fail to arrest in response to a wide variety of DNA damaging agents. This has been shown to be due to the stabilization of the p53 protein and enhancement of its transcriptional activity leading to arrest at both $G_2$/M phases, possibly through

transactivation of the 14-3-3 proteins (Hermeking et al., 1997 *Mol. Cell*, 1, p. 3-11), and at $G_1$/S, through up-regulation of $p21^{Waf1/Cip1}$ (Brugarolas et al., 1995 *Mol. Cell*, 1, p. 3-11; and Deng et al., 1995: *Cell*, 82, p. 675-684). In light of this data, there may exist not only cross-talk between p53 and transcription of $p21^{Waf1/Cip1}$, but also an influence of the p53 protein on whether $p21^{Waf1/Cip1}$ is growth inhibitory, or required for growth through an unknown mechanism.

In summary, the above Example II demonstrates that the permissive effect of the CKI $p21^{Waf1/Cip1}$, which was unequivocally demonstrated in A10 VSM cells, does not occur under similar conditions in A431 cells. Since the principle difference in the growth factor mitogenic signaling cascades between these two cell lines relates to a mutant and inactive p53 in the A431 cells, it is believed that the permissive effect of $p21^{Waf1/Cip1}$ requires the presence of active p53 protein.

## EXAMPLE III

**Antisense $p21^{Waf1/Cip1}$ Potentiates Ionizing Radiation- and Chemotherapy-induced Cell Cycle Arrest in VSM Cells**

**Materials**: PDGF-BB and mouse monoclonal anti-human $p21^{Waf1/Cip1}$ were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-human caspase-3 antibody and anti-goat horseradish peroxidase-conjugated IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectin® was obtained from Life Technologies (Rockville, MD). Reagents for the Enhanced Chemiluminescence system and [$^3$H]thymidine were obtained from Amersham (Arlington Heights, IL). Adriamycin (doxorubicin) was obtained from Pharmacia & Upjohn (Kalamazoo, MI). All other reagents, including Hoechst 33258, were from Sigma Chemical Co. (St. Louis, MO).

**Cell culture and DNA synthesis assays**: Cultures of A10 aortic VSM and A431 sarcoma cells were obtained from American Type Culture Collection (Rockville MD), and were maintained as described (Weiss RH, et al. *Am J Physiol* 274:C1521-C1529, 1998); the

A10 cells were used between passages 15 and 25. Where indicated, the cells were growth-arrested by placing them in serum-free quiescence medium, exposed to PDGF-BB or 10% serum-containing medium as indicated, and [$^3$H]thymidine incorporation was assessed as previously described (Weiss RH, Nuccitelli R: *J Biol Chem* 267:5608-5613, 1992).

**Ionizing radiation experiments**: Cells were transfected with oligodeoxynucleotide 16-24 hours prior to γ-irradiation. Cells were subjected to 1-12 Gy of γ-irradiation from a $^{137}$Cs source. 30 minutes after irradiation cells were stimulated with PDGF-BB or 10% serum media. After 6 hours of stimulation, cells received 1 μCi [$^3$H]thymidine per ml of media and were analyzed for DNA synthesis. For Western blots, cells were lysed 4 hours after irradiation unless stated otherwise.

**Antisense transfections**: Phosphorothioate antisense and random sequence control oligodeoxynucleotides were synthesized by Oligodeoxynucleotides Etc. (Wilsonville, OR). The $p21^{Waf1/Cip1}$ antisense vector was designed around the start codon of rat $p21^{Waf1/Cip1}$, with sequence 5'-GAC ATC ACC AGG ATC GGA CAT-3' (SEQ. ID NO.:1). The scrambled random sequence control oligodeoxynucleotide was 5'-TGG ATC CGA CAT GTC AGA-3' (SEQ. ID NO.:3). For the lipofection procedure, cells were grown to 60% confluence, washed with sterile phosphate-buffered saline, and the appropriate concentration of oligodeoxynucleotide was mixed with 6.6 μL of Lipofectin® per ml of Opti-MEM medium and was added to the cells for 4 h at 37°C. Serum-free medium (without oligodeoxynucleotide) was added overnight, the media was changed in the morning and the cells were stimulated as indicated.

**Western blots**: Cells were grown to confluence in 6 cm culture dishes and serum deprived. After transfection and or treatment with appropriate agonist, conditioned medium was removed and saved and the cells were washed with phosphate-buffered saline and lysed in lysis buffer. Both supernatant and cell lysate were normalized to the lysate protein concentrations and Western blotted as described (Weiss RH, et al. *Am J Physiol* 274:C1521-C1529, 1998).

**Apoptosis assays:** Cells were grown on collagen-coated coverslips, and transfected as stated above. 16-24 hours after transfection, cell were exposed to 12 Gy dose of γ-irradiation or 0.5 μM wortmannin. 24 hours later, cells were fixed in 3.7% formaldehyde (diluted in PBS) for 10 minutes. The cells were rinsed with cold PBS and permeabilized using 0.1% Triton X-100 (diluted in deionized water) for 5 minutes. Rinsed again with PBS, the cells on the coverslips were submerged in Hoechst Staining solution (3.0 μl in 37.5 ml deionized water) for 5 minutes. Cells were given a final three rinses with cold PBS before being mounted in polyvinyl alcohol mounting medium. Cell nuclei were visualized using a ZEISS WL Microscope and photographed under 40x.

1. Effect of Ionizing Radiation and Adriamycin on Growth of VSM Cells

Both ionizing radiation and Adriamycin cause growth arrest due to induction of p53 as a result of DNA damage. This tumor suppressor protein sets in motion a series of events culminating in cell cycle arrest in G1 and G2 (Bunz F, et al. *Science* 282:1497-1501, 1998; Agarwal ML, et al. *Proc Natl Acad Sci U S A* 92:8493-8497, 1995; and Poon RC, et al. *J Biol Chem* 271:13283-13291, 1996). Examples I and II utilized two established mesenchymal-derived cell lines, one (A10 VSM cells) possessing intact p53 and the other (A431 squamous carcinoma cells) which has a mutant p53 gene and an inactive p53 protein (Kwok TT, et al. *Cancer Res* 54:2834-2836, 1994). In order to characterize the response of these particular cells to DNA damage, growth of these cells in various stages of the cell cycle was examined. Upon removal of serum, "normal" cells generally remained in G0, and subsequent stimulation with serum or growth factors caused them to resume transit through the cell cycle. To set the stage for further studies, the cells were examined under four conditions: (1) when left in serum-containing medium; (2) when left in serum-free medium; (3) when stimulated with complete medium after serum-free medium, and (4) when stimulated with PDGF-BB after serum-free medium.

[$^3$H]thymidine incorporation was examined, which is a measure of transit through S phase of the cell cycle, after the cells were exposed to irradiation. A10 VSM cells were growth arrested after exposure to ionizing radiation under all conditions examined, likely

through p53-mediated induction of p21 and subsequent cdk inhibition (Figure 14A). In A431 cells, which possess a mutant and inactive p53, ionizing radiation failed to cause consistent inhibition of DNA synthesis, although there was slight (but significant) inhibition in cells which were exposed to serum after serum starvation (Figure 14B). This suggested that p53, and its downstream effector p21, are important in mediating the cell cycle arrest and DNA damage repair in cells exposed to ionizing radiation. Subsequent experiments in VSM cells were therefore performed in cells that were left in complete medium, since these were the conditions under which the changes with radiation were maximal, and because these conditions most closely replicate the *in vivo* milieu.

The above findings (Figure 14 A and B) suggest that p53 status is essential to determining the response of cells to ionizing radiation and, by extension, to DNA damage. This is consistent with data reported by other investigators, as the level of p53 has been shown to be a very sensitive indicator of DNA damage. It has been suggested that one double-stranded DNA break is sufficient to induce this protein (Di Leonardo A, et al. *Genes Dev* 8:2540-2551, 1994). Furthermore, both ionizing radiation and Adriamycin are known to cause DNA damage, and have been shown to increase p53 expression in order to mediate G1 and G2 arrest, such that DNA repair can occur (Bunz F, et al. *Science* 282:1497-1501, 1998; and Agarwal ML, et al. *Proc Natl Acad Sci U S A* 92:8493-8497, 1995). The downstream effector of p53 is p21, and since p21 has been shown to have variable effects on cell cycle events (Sherr CJ, Roberts JM. *Genes and Dev* 13:1501-1512, 1999) and on growth (Weiss RH, et al. *J Biol Chem* 275:10285-10290, 2000; Weiss, R. H. and Randour, C. *Cellular Signalling*, 12:413-418, 2000), whether p21 attenuation affects cell growth in response to DNA damage, was studied.

2. Effect of $p21^{Waf1/Cip1}$ Antisense Oligonucleotide (SEQ. ID NO.:1) on Cell Growth in Response to DNA Damage

To confirm that p21 levels were increased after exposure to ionizing radiation, and that the oligonucleotides inhibit this phenomenon in A10 cells, p21 protein levels under these conditions were assayed. The level of p21 was increased after ionizing radiation (12 Gy)

up to 4 h (Figure 15A). p21 levels were consistently low in all cells transfected with the antisense oligonucleotide to p21 and then stimulated with ionizing radiation (12 Gy). No peak of induction was observed in these cells (Figure 15B). In the cells transfected with the random sequence control oligonucleotide, there was initial slight inhibition of p21 levels at ½ and 1 h after ionizing radiation, but the levels rapidly increased by 4 h (Figure 15b). With the exception of the initial decrease in p21 levels at ½ and 1 h, the results with the control oligonulceotides were similar to that observed in non-transfected cells. The specificity of the antisense oligonucleotide was previously demonstrated by showing no change in expression of α-actin in this cell line after antisense p21 oligonucleotide transfection (Weiss RH, et al. J Biol Chem 275:10285-10290, 2000; Weiss, R. H. and Randour, C. *Cellular Signalling*, 12:413-418, 2000).

Adriamycin is a prototypical DNA damaging agent used in the treatment of a variety of cancers. The predominantly G2 arrest seen after Adriamycin treatment has been associated with an increase in p21 levels in some cell lines (Siu WY, et al. *FEBS Lett* 461:299-305, 1999). In order to determine whether similar augmentation of p21 is seen in A10 VSM cells and whether the antisense p21 oligonucleotide is inhibiting this response, these cells were treated with Adriamycin either with or without first transfecting the cells with the antisense and control oligonucleotides. Confluent VSM cells were transfected with antisense oligonucleotide to p21 or random sequence control oligonucleotide as described above. After 24 h, the cells were exposed to Adriamycin (500 ng/ml), lysed at the indicated times after exposure, and Western blotted with p21 antibody. As in the case of cells exposed to ionizing radiation, p21 levels were increased by Adriamycin at similar times, with a marked attenuation of this response in cells transfected with the antisense p21 oligonucleotide(Figure 16).

3. Effect of the Levels of p21 on Growth Activity

Having successfully inhibited the peak of p21 expression in VSM cells after ionizing radiation as well as Adriamycin exposure, it was determined whether growth modulation,

after these maneuvers, is altered after suppression of the peak p21 level, which occurs in normal cells when so stimulated.

Confluent VSM cells were transfected with the antisense oligonucleotide to p21 (SEQ. ID NO.:1) (or a random sequence control oligonucleotide(SEQ. ID NO.:3) and exposed to various doses of ionizing radiation. After 24 h, the cells were exposed to the indicated dose of radiation. Six hours later, [$^3$H]thymidine was added overnight and DNA synthesis was assessed as in Figure 14. Cells transfected with the control oligonucleotide (SEQ. ID NO.:3) showed attenuation of DNA synthesis as a function of radiation dose from 1 to 12 Gy (Figure 17). After transfection of the cells with the antisense p21 oligonucleotide, there was a marked inhibition of DNA synthesis in non-irradiated cells, as previously shown in Example I (Weiss RH, et al. *J Biol Chem* 275:10285-10290, 2000). The effects of irradiation on cell cycle arrest were potentiated at higher doses of radiation, with a maximum potentiation at 8 Gy. The potentiation at 12 Gy did not reach statistical significance, probably due to cell mortality at that dose.

4. Effect of Antisense Oligonucletide-Mediated Inhibition of p21 on Adriamycin Induced Cell Cycle Arrest

It has been previously shown that p21 (-/-) cells are more sensitive to the killing effects of a variety of chemotherapeutic agents (Waldman T, et al. *Nature* 381:713-716, 1996; Stewart ZA, et al. *Cancer Res* 59:3831-3837, 1999; Waldman T, et al. *Nat Med* 3:1034-1036, 1997; and Fan S et al. *Oncogene* 14:2127-2136, 1997). The effect of antisense oligonucleotide-mediated inhibition of p21 on Adriamycin induced cell cycle arrest was determined. Confluent VSM cells were transfected with control (SEQ. ID NO.:3) or antisense p21 oligonucleotides (SEQ. ID NO.:1) and exposed to Adriamycin at concentrations from 500 to 2000 ng/ml. After 24 h, the cells were exposed for 2 h to the indicated concentration of Adriamycin. Two hours later, [$^3$H]thymidine was added overnight and DNA synthesis was assessed as in Figure 14. While the growth inhibitory effect of Adriamycin on control oligonucleotide transfected cells plateaued in this range,

there was significant potentiation of growth inhibition in antisense p21 oligonucleotide transfected cells when exposed to from 1000 to 2000 ng/ml Adriamycin (Figure 18).

5. Effect of Antisense Oligonucletide-Mediated Inhibition of p21 on Apotosis

While it was shown that p21 is required for serum and PDGF-stimulated growth in VSM cells (Weiss RH, et al. *J Biol Chem* 275:10285-10290, 2000), there are also reports that the absence of p21 may cause cells to be converted from growth arrest to apoptosis (Tian H, et al. *Cancer Res* 2000 Feb 1;60 (3):679 -84 60:679-684, 2000).

In order to determine whether the potentiation of ionizing radiation and Adriamycin induced DNA synthesis inhibition by p21 antisense oligonucleotides is accompanied by apoptosis, caspase-3 activation in response to ionizing radiation was examined. Caspase-3 is an effector caspase whose activation (leading to apoptosis) results in a 20 kD cleavage product which can be assessed by Western blotting (McCarthy NJ, Evan GI. *Curr Top Dev Biol* 36:259-278, 1998). This process is an early event in a cascade of reactions which ultimately leads to apoptosis, as is evident by the fact that inactivation of caspase-3 dramatically reduces apoptosis in several cell lines (Woo M, et al. *Genes Dev* 12:806-819, 1998).

VSM cells were transfected with antisense or control oligonucleotides overnight and then stimulated with ionizing radiation. While ionizing radiation alone did not induce caspase-3 activation at 4 h at radiation doses of 12 Gy, there was an impressive increase in caspase-3 activation in cells transfected with antisense p21 oligonucleotide, either alone or after exposure to ionizing radiation (Figure 19A).

Adriamycin increases p53 and p21 resulting in cell cycle arrest (Siu WY, et al. *FEBS Lett* 461:299-305, 1999). As in the case of ionizing radiation, caspase-3 activation was observed in cells transfected with antisense p21 (SEQ. ID NO.:1), but not random sequence oligonucleotides (SEQ. ID NO.:3), with little effect of Adriamycin alone on this apoptosis effector at the time examined (Figure 19b).

As another measure of apoptosis, transfected cells fixed and stained with Hoechst 33258 were examined. Cells transfected with antisense p21 (SEQ. ID NO.:1), but not random sequence control oligonucleotides (SEQ. ID NO.:3), showed extensive apoptotic changes 24 h after transfection (Figure 20).

The results described in this Example show apoptosis, as well as potentiation of cell cycle arrest in VSM cells using a simple and straightforward technique, suggesting a new paradigm for treatment of fibrotic diseases, such as angioplasty restenosis, hemodialysis graft stenosis, mesangial proliferative glomerular disease, as well as in most pathogenic models of atherosclerosis. Indeed, the findings that transfection of the antisense p21 oligonucleotide alone induced apoptosis, coupled with the findings from other investigators that p21 (-/-) mice do not have phenotypic changes or an increased susceptibility to spontaneous tumors (Deng C, et al. *Cell* 82:675-684, 1995), suggests the use of antisense p21 oligonucleotides as cancer therapeutics (see Tian H, et al. *Cancer Res* 2000 Feb 1;60 (3):679 -84 60:679-684, 2000).

While the CKIs had been considered to be solely growth inhibitory, data is emerging that these molecules in fact have both positive and negative effects on cell cycle checkpoints (Sherr CJ, Roberts JM. *Genes and Dev* 13:1501-1512, 1999) as well as on cell growth (Weiss RH, et al. *J Biol Chem* 275:10285-10290, 2000). Furthermore, since p21 arrests transit through the cell cycle at G1 in order for DNA damage repair to occur (Bunz F, et al. *Science* 282:1497-1501, 1998), disruption of this checkpoint in p21(-/-) cells results in multiploidy and subsequent targeting of the cells for apoptosis (Waldman T, et al. *Nature* 381:713-716, 1996; and Mantel C, et al. *Blood* 93:1390-1398, 1999).

The above results demonstrate that using a novel antisense oligonucleotide corresponding to the translational start site of the p21 gene to attenuate p21 levels (Example I), arrest of the cell cycle and apoptosis occurs in VSM cells which have had p21 levels reduced using p21 inhibitory agents. Furthermore, under these conditions cell cycle inhibitory responses to radiation, and the DNA damaging chemotherapeutic agent, Adriamycin, are both potentiated.

While use of antisense oligonucleotides to p21 demonstrated a clear potentiation of apoptosis, after exposure of the cells to DNA damaging agents, it was surprising that caspase-3 activation did not occur with either radiation or Adriamycin alone at the times examined, despite inhibition of cell cycle transit as assessed by DNA synthesis. While not wishing to be bound by any particular theory, it is possible that caspase-3 was cleaved at a later time that was not apparent in the gels examined. In any case, the propensity of the antisense p21 oligonucleotide to initiate apoptosis and lead to its morphological characteristics in VSM cells is abundantly clear from these results.

The application of p21 inhibitors in renal disease, in addition to their role as vascular cell growth attenuators, has become evident in a recent study showing that p21(-/-) mice, as compared to wild type, were less likely to develop chronic renal failure after renal ablation (Megyesi J, et al. *Proc Natl Acad Sci U S A* 96:10830-10835, 1999). While these investigators suggested that this effect was due to their finding that the absence of the p21 gene leads to a more hyperplastic response, apoptosis of mesangial or other renal cells may also be contributory. Other renal investigators have shown that diabetic p21(-/-) mice do not develop the same degree of glomerular hypertrophy as their wild type counterparts (Al Douahji M, et al. *Kidney Int* 56:1691-1699, 1999), an effect which may, in light of the data herein, also be due to apoptosis of glomerular cells.

The likelihood that antisense oligonucleotides may have potential therapeutic utility is further bolstered by experiments in animal models. Data has been generated on the pharmacology of antisense oligonucleotides in animal models. For example, the acute $LD_{50}$ of phosphorothioates is 500 mg/ml (Crooke, S. T. *Therapeutic applications of oligonucleotides.* 1995. Austin, R.G.Landes), well above the effects seen herein at nanomolar quantities of p21 antisense oligonucleotides. Furthermore, phosphorothioate oligonucleotides are rapidly and extensively absorbed after intravenous administration in rats, and distribute broadly to all peripheral tissues, especially liver, kidney, bone marrow, skeletal muscle and skin (Crooke, S. T. *Therapeutic applications of oligonucleotides.* 1995. Austin, R.G.Landes). An antisense oligonucleotide to c-*raf* injected into nude mice implanted with human tumors showed decent tissue uptake

without the benefit of lipofection reagents (Monia BP, et al. *J Biol Chem* 267:19954-19962, 1992. The data presented herein showing the effect of the novel antisense cyclin kinase inhibitors of the invention in vascular cells support the use of the compositions and methods of the invention in the treatment of vascular and renal proliferative diseases.

5

EXAMPLE IV

**The Effect of $p21^{Waf1/Cip1}$ on TBF-β-Mediated Matrix Protein Secretion**

10 **Materials**: TGF–β1 and mouse monoclonal anti-human $p21^{Waf1/Cip1}$ were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-rat fibronectin and laminin antibodies were obtained from Chemicon (Temecula, CA). Anti-goat horseradish peroxidase-conjugated IgG was obtained from BioRad (Richmond, CA). Lipofectin® was obtained from Life Technologies (Rockville, MD). Reagents for the Enhanced
15 Chemiluminescence system and [$^3$H]thymidine were obtained from Amersham (Arlington Heights, IL). All other reagents, including mouse monoclonal α-actin antibody and protein A-Sepharose beads, were from Sigma Chemical Co.(St. Louis, MO).

20 **Cell culture, DNA synthesis, and proliferation assays**: Cultures of A10 aortic VSM cells were obtained from American Type Culture Collection (Rockville MD), were maintained as described (Weiss, R. H.; et al. *Am.J.Physiol.* 274: C1521-C1529; 1998), and were used between passages 15 and 25. The cells were growth-arrested by placing them in serum-free quiescence medium, exposed to TGF–β or 10% serum-containing
25 medium as indicated in the figures, and [$^3$H]thymidine incorporation was assessed as previously described (Weiss, R. H.; Nuccitelli, R. *J. Biol. Chem.* 267: 5608-5613; 1992).

**Oligodeoxynucleotide transfections**: Phosphorothioate antisense and random sequence control oligodeoxynucleotides were synthesized by Oligonucleotides Etc. (Wilsonville,
30 OR). The $p21^{Waf1/Cip1}$ antisense vector was designed around the start codon of rat $p21^{Waf1/Cip1}$, with sequence 5'-GAC ATC ACC AGG ATC GGA CAT-3' (SEQ. ID

NO.:1). The scrambled random sequence control oligodeoxynucleotide was 5'-TGG ATC CGA CAT GTC AGA-3' (SEQ. ID NO.:3). For the lipofection procedure, cells were grown to 60% confluence, washed with sterile phosphate-buffered saline, and the appropriate concentration of oligodeoxynucleotide was mixed with 6.6 μL of Lipofectin® per ml of Opti-MEM medium and was added to the cells for 4 h at 37°C. Serum-free medium (without oligodeoxynucleotide) was added overnight, the media was changed in the morning, and the cells were stimulated with TGF–β or serum as indicated.

**Immunoprecipitations:** VSM cells were grown to confluence. After incubation under appropriate conditions, the cells were washed with ice-cold phosphate–buffered saline and immediately lysed in lysis buffer (20 mM Tris [pH7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml Leupeptin, 1 mM PMSF) at 4°C. The cells were scraped off with a rubber spatula and the insoluble material removed by centrifuging at 10,000x g for 10 min at 4°C. Protein concentration was determined by $A_{595}$, and lysates containing equal amounts of protein were incubated with 4 ml anti-fibronectin antibody at 4°C overnight. Protein A-Sepharose beads were added and the resulting mixture was incubated for an additional 2 h at 4°C. The beads were centrifuged in a microfuge for 20 sec, and the pellet was washed 3 times with cold lysis buffer. The supernatant was decanted, gel loading buffer was added to the precipitate, and the solution was boiled for 5 min, and centrifuged. The supernatant was electrophoresed on a 7.5% SDS-polyacrylamide gel with equal volumes of sample per lane. The proteins were electrophoretically transferred to nitrocellulose and probed with fibronectin antibody.

**Western blots:** Cells were grown to confluence in 6 cm culture dishes and serum deprived. After transfection and/or treatment with appropriate agonist, conditioned medium was removed and saved and the cells were washed with phosphate-buffered saline and lysed in lysis buffer. Both supernatant and cell lysate were normalized to the lysate protein concentrations and Western blotted as described (Weiss, R. H.; et al. *Am.J.Physiol.* 274: C1521-C1529; 1998).

RESULTS

TGF–β has a bimodal effect on mitogenesis, being stimulatory or inhibitory depending on cell confluency and cell type (Moses, H. L.; et al. *Cell* 63: 245-247; 1990; and Centrella, M. et al. *J. Biol. Chem.* 262: 2869-2874; 1987). However, TGF–β is largely growth inhibitory *in vivo* (reviewed in Moses, H. L.; et al. *Cell* 63: 245-247; 1990). This property was confirmed in early passage rat VSM cells (Weiss, R. H.; et al. *Kidney Int.* 48: 738-744; 1995) and late passage rat mesangial cells (Weiss, R. H.; Ramirez, A. *Nephrol. Dial. Transplant.* 13: 2804-2813; 1998). To determine whether A10 VSM cells behave similarly, these cells were serum-starved for 24 h prior to stimulation with from 0.1 to 10 ng/ml TGF–β, and their ability to incorporate [$^3$H]thymidine into DNA was examined (expressed as mean ± s.e.m. of three wells per data point). At all concentrations tested, DNA synthesis was significantly reduced with the addition of TGF–β for 24 h (Figure 21). To determine whether this growth inhibitory effect persisted when the A10 VSM cells were stimulated to enter $G_1$ with the addition of serum, the effect of the addition of 10% serum-containing media on TGF–β-exposed cells at the same concentrations was examined and found to exhibit similar growth-inhibitory results (Figure 22). DNA synthesis was assessed by [$^3$H]-thymidine incorporation and is expressed as mean ± s.e.m. of three wells per data point; absolute counts differ slightly from other experiments due to differences in starting confluency of the cells.

Using a random sequence phosphorothioate oligodeoxynucleotide (SEQ. ID NO.:3) as a control, the effect of the antisense oligodeoxynucleotide to p21 (400 nM) on p21 and α-actin protein expression in A10 VSM cells, was examined. The lysates, normalized for protein content, were Western blotted with either p21 or α-actin antibody. There was marked inhibition of p21 protein level, but no effect on the level of α-actin protein level, after transfection of 400 nM antisense p21 oligodeoxynucleotide (SEQ. ID NO.:1) (Figure 23). Thus, this antisense oligodeoxynucleotide is specific in its inhibition of p21

protein expression and would not be expected to directly inhibit transcription or translation of matrix proteins which, of course, are unrelated in sequence to the CKIs.

1. Effect of Transfection of Antisense p21 (SEQ. ID NO.:1) or random sequence (SEQ. ID NO.:3) oligodeoxynucleotide on TGF–β

Cells were transfected with the appropriate oligodeoxynucleotide (antisense or random sequence) for 4 h. The cells were then serum-starved overnight and subsequently stimulated with 10% serum-containing media. Two hours after stimulation with serum, TGF–β was added at concentrations from 0.1 to 10 ng/ml for 24 h. While DNA synthesis of cells transfected with antisense p21 was markedly inhibited relative to cells transfected with control oligodeoxynucleotide (as it was previously shown when comparing antisense p21 with sense p21 oligodeoxynucleotide (Example 1, herein), the inhibitory effect of TGF–β at 10 ng/ml was still present under both conditions (Figure 24).

2. Effect of p21 on Matrix Protein Secretion in VSM Cells

The p21 influences on matrix protein secretion in VSM cells were determined. Levels of the matrix proteins laminin and fibronectin were examined in both TGF–β stimulated lysate and conditioned media of cells in which p21 expression had been attenuated. The cells were transfected with either antisense p21 (SEQ. ID NO.:1) (400 nM), or random sequence control (SEQ. ID NO.:3) (400 nM) oligodeoxynucleotide, and then stimulated with TGF–β at for 0.1 to 10 ng/ml for 4 h. Conditioned media and cell lysate were collected, and both medium and lysate volumes were normalized for the protein content in total cell lysate to exclude any skewing of the data due to cell proliferation. The proteins were electrophoresed and immunoblotted with either fibronectin (after immunoprecipitation to eliminate extraneous bands which appeared in the absence of this procedure) or laminin antibody. Production and secretion of laminin was markedly reduced after attenuation of p21, yet the effect of TGF–β was to decrease laminin secretion at higher doses in control oligodeoxynucleotide transfected cells (Figure 25). Fibronectin production and secretion into the medium was similarly decreased after p21

attenuation, yet, in this case, TGF–β induced fibronectin production with a maximal level in lysate when fibronectin was administered at higher doses (Figure 26).

DISCUSSION

The early lesions of atherosclerosis are associated with migration and proliferation of VSM cells. Once these cells enter the proliferative state, they attain a synthetic phenotype which causes them to secrete matrix proteins (Assoian, R. K.; Marcantonio, E. E. *J. Clin. Invest* 100: S15-S18; 1997; and Thyberg, J.; et al. *Arteriosclerosis* 10: 966-990; 1990). Exuberant secretion of these proteins may lead to fibrosis, but the same proteins may also regulate the cell phenotype and cause it to either remain secretory or become proliferative (Thyberg, J et al. *J. Histochem. Cytochem.* 45: 837-846; 1997).

Matrix proteins are secreted by a variety of cells and are important for structural integrity in the normal environment, yet these same proteins may be detrimental when they occur in abundance in the disease setting (reviewed in (Rizzino, A. *Dev. Biol.* 130: 411-422; 1988)). Furthermore, matrix proteins have been assigned the role of cell cycle control elements in atherosclerotic disease (Assoian, R. K.; Marcantonio, E. E. *J. Clin. Invest* 100: S15-S18; 1997). The specific matrix proteins laminin and fibronectin are important in modulating the switch from contractile to synthetic phenotype in VSM cells (reviewed in (Thyberg, J.; et al. *Arteriosclerosis* 10: 966-990; 1990)).

The extracellular matrix plays a key role in the progression of fibrosis in a variety of disparate diseases in multiple organ systems. In VSM and related glomerular mesangial cells, overexuberant secretion of matrix proteins is likely responsible for progression of atherosclerosis as well as of glomerular disease (reviewed in (Assoian, R. K.; Marcantonio, E. E. *J. Clin. Invest* 100: S15-S18; 1997; and Border, W. A.; Noble, N. A. *N. Engl. J. Med.* 331: 1286-1292; 1994)). The growth factor TGF–β, which in VSM cells is generally growth inhibitory (Weiss, R. H.; et al. *Kidney Int.* 48: 738-744; 1995; and Reddy, K. B.; Howe, P. H. *J. Cell. Physiol.* 156: 48-55; 1993), causes fibrosis in a variety of tissues and has been linked to an increase in matrix protein production as an etiology

for this pathologic process (Border, W. A.; et al. *Kidney Int.* 37: 689-695; 1990; and Nakamura, T.; et al. *Kidney Int.* 41: 1213-1221; 1992), such that antibodies to TGF–β1 suppress arterial intimal hyperplasia and restenosis (Wolf, Y. G.; et al. *J. Clin. Invest* 93: 1172-1178; 1994) as well as experimental glomerulonephritis (Border, W. A.; et al. *Nature* 346: 371-374; 1990).

Studies have shown that the CKI p27 may mediate the switch from hyperplasia to the hypertrophic phenotype in VSM cells in response to TGF–β (Gibbons, G. H.; et al. *J. Clin. Invest.* 90: 456-461; 1992 Braun-Dullaeus, R. C.; et al. *J. Clin. Invest.* 104: 815-823; 1999)). Further, CKIs are important in regulating cell cycle transit (Sherr, C. J.; Roberts, J. M.. *Genes and Dev.* 13: 1501-1512; 1999). Thus it was decided to examine whether the CKI p21 plays a role in the regulation of TGF–β-mediated matrix protein synthesis and secretion in VSM cells.

TGF–β is a growth factor that has variable influences on VSM and glomerular mesangial cells, the latter of which are modified smooth muscle cells. Depending on the cell type and culture conditions, TGF–β can be either stimulatory or inhibitory towards cell growth (Moses, H. L.; et al. *Cell* 63: 245-247; 1990). Despite its bimodal effect on cell proliferation, it is clear that TGF–β induces the pathologic appearance of matrix proteins, and thus this growth factor has been implicated as a causative agent in a variety of diseases which are characterized by fibrosis (Border, W. A.; Noble, N. A. *N. Engl. J. Med.* 331: 1286-1292; 1994; and Border, W. A.; et al. *Kidney Int.Suppl* 49: S59-S61; 1995). There is even evidence that fibronectin (Madri, J. A.; et al. *J.Cell Biol.* 106: 1375-1384; 1988) and laminin (Thyberg, J et al. *J. Histochem. Cytochem.* 45: 837-846; 1997) may actually be mediating the growth inhibitory effects of TGF–β. In any case, an understanding of the mechanism by which vascular cells attain the secretory phenotype, and can therefore be influenced by TGF–β to secrete matrix proteins, is of pivotal importance in the study of fibrotic diseases.

The results described herein show that p21 antisense oligodeoxynucleotides, which specifically inhibit p21 protein levels in these cells, markedly attenuate both synthesis

and secretion of the matrix proteins fibronectin and laminin. These results in vascular cells have profound implications for treatment of fibrotic diseases, such as atherosclerosis and restenosis. Furthermore, since glomerular mesangial cells are modified VSM cells, the findings can be extended to treatment of glomerular diseases characterized by fibrotic changes mediated through matrix protein production.

The CKIs p21 and p27 have long been known to be induced by TGF–β (Polyak, K.; et al. *Genes Dev.* 8: 9-22; 1994; and Grau, A. M.; et al. *Cancer Res.* 57: 3929-3934; 1997), and it has been assumed that expression of those proteins links this growth factor to cell cycle arrest. It has been shown that in VSM, mesangial, and prostate carcinoma cells, p27 and p21 are induced by the growth inhibitory statins (Weiss, R. H.; Ramirez, A.; Joo, A. *J. Am. Soc. Nephrol.* 9: 1880-1890; 1999; Lee, S. J.; et al. *J. Biol. Chem.* 273: 10618-10623; 1998; and Terada, Y.; et al. *J. Am. Soc.Nephrol.* 9: 2235-2243; 1999). While these proteins likely in some manner cause attenuation of DNA synthesis in that setting, it has also been shown that p21 is required for the full mitogenic effect of PDGF and serum (Weiss, R. H.; et al. *J. Biol. Chem.* 275: 10285-10290; 2000; Weiss, R. H. and Randour, C. *Cellular Signalling*, 12:413-418, 2000), a correlation which has recently been confirmed by another group (Wakino, S, et al. *J Biol Chem* 275(9):22435-22441,2000). Other investigators have shown the existence of a switch from a contractile to a synthetic phenotype in cells stimulated to proliferate (Thyberg, J.; et al. *Arteriosclerosis* 10: 966-990; 1990). The data presented in Example IV are consistent with this scenario, since decreased synthesis of matrix protein in cells transfected with antisense p21 was shown, concomitant with attenuation of proliferation in these cells.

Example IV demonstrates that the ability of TGF–β to synthesize and secrete the matrix proteins laminin and fibronectin, but not TGF–β's entire growth inhibitory effect, is mediated through p21. It was previously shown that, in mesangial cells, TGF–β at 10 ng/ml induced secretion of the matrix proteins fibronectin and laminin (Weiss, R. H.; Ramirez, A. *Nephrol. Dial. Transplant.* 13: 2804-2813; 1998). However, in the VSM cells used in the present experiments, higher concentrations of TGF–β were associated with decreased laminin synthesis and secretion into conditioned media. This may be due

to the fact that continued stimulation of VSM cells by TGF–β causes them to remain in a synthetic phenotype associated with fibronectin secretion, whereas cells remain in the contractile phenotype when grown in the presence of the secreted laminin (Thyberg, J et al. *J. Histochem. Cytochem.* 45: 837-846; 1997).

It is noteworthy that transfection of antisense oligodeoxynucleotides has been shown to be effective *in vivo* both with and without lipofection reagents, and in some situations antisense oligodeoxynucleotides have even proved effective under conditions as simple as intravenous oligodeoxynucleotide infusions (Monia, B. P.; et al. *Nat. Med.* 2: 668-675; 1996). Therefore, these compounds may be useful clinically to target molecules important in fibrotic diseases such as, atherosclerosis, restenosis and glomerular disease. .

It has been demonstrated that, in the case of mesangial cells, p21 is required for glomerular hypertrophy in experimental diabetic nephropathy (Al Douahji, M.; et al. *Kidney Int.* 56: 1691-1699; 1999). p21 maybe allowing secretion of matrix proteins in this scenario such that its inhibition would diminish this response. In VSM cells, p27 has been shown to have a similar role in the promotion of hypertrophy (Braun-Dullaeus, R. C.; et al. *J. Clin. Invest.* 104: 815-823; 1999), yet the role of p21 and matrix protein secretion in this phenomenon in VSM cells is not known. In the setting of angioplasty, VSM cells have been shown to modulate from a contractile to a synthetic phenotype after induction of intimal lesions by balloon catheterization (Grunwald, J.; et al. *Exp. Mol. Pathol.* 46: 78-88; 1987; and Manderson, J. A.; et al. *Arteriosclerosis* 9: 289-298; 1989). This may in turn result in excess production of matrix proteins, leading to ultimate restenosis of the vessel. Attenuation of matrix protein production and secretion with antisense p21 transfection into VSM cells may therefore prove to be useful, where such oligodeoxynucleotides could be lipofected into angioplastied blood vessels at the time of balloon catheterization.

A study was performed in cells from knockout mice, where it was shown that lack of a functional p21 gene ameliorated progression to chronic renal failure (Megyesi, J.; et al. *Proc. Natl. Acad. Sci.U.S.A.* 96: 10830-10835; 1999). In this study, none of the p21(-/-)

mice developed glomerulosclerosis or interstitial fibrosis, as opposed to 70% of the glomeruli in p21 (+/+) animals, suggesting that p21 may be responsible for mediating a TGF–β effect on the cells leading to fibrosis by means of matrix protein secretion.

## EXAMPLE V

***In Vivo* Studies of the Effect of Antisense Oligonucletide of p21 on Met-1 breast cancer**

Angiogenesis is the means by which growing tumors maintain oxygen necessary for their survival by means of their creation of auxiliary blood vessels. This is generally thought to result for the effect of various growth factors (such as VEGF) acting on endothelial cells. The possibility that angiogenesis may also be attenuated by inhibition of VSM cells is a possibility which has not been adequately investigated.

**Materials and Methods:** NZW mice were obtained and injected subcutaneously 2 days after their arrival with Met-1 breast cancer cells. Each mouse was injected on two sides of the breast area, such that two tumors arose in most animals. The following day, intraperitineal injections at the indicated concentrations were made on a daily basis. When tumors appeared, they were measured in two dimensions using calipers and area of the tumors was used as rough measure of tumor mass. The tumor areas were averaged for each mouse and then these numbers were subsequently averaged. The general health of the mice was monitored on a daily bases as well.

**Antisense Preparation:** Phosphorothioate antisense oligodeoxynucleotides were synthesized by Oligonucleotides Etc. (Wilsonville, OR). The $p21^{Waf1/Cip1}$ antisense vector was designed around the start codon of rat $p21^{Waf1/Cip1}$, with sequence 5'-GAC ATC ACC AGG ATC GGA CAT-3' (SEQ. ID NO.:1). The sense $p21^{Waf1/Cip1}$ sequence is 5'-ATG TCC GAT CCT GGT GAT GTC-3' (SEQ. ID NO.:2). The scrambled random sequence control oligodeoxynucleotide was 5'-TGG ATC CGA CAT GTC AGA-3' (SEQ. ID NO.:3).

Animal models of potential chemotherapeutics using the p21 antisense oligonucleotides of the invention were examined. Intraperitoneal injection of the oligonucleotides into mice previously injected with cells from a Met-1 breast cancer line (Bourguignon, L. Y. et al, *J. Cell Phys.* 1998, 176, 206-215; Lau, D.H. et al., *Cancer Biother. Radiopharm.* 1999, 14, 31-6) were studied. The p21 antisense oligonucleotide or random sequence control oligonucleotide at a concentration of 0.6 mg oligonucleotide/kg mouse (Monia, B. P.; et al. , 1996, *Nat. Med.* 2: 668-675)were injected intraperitoneally daily when tumors first became palpable (day 1). There were 2 tumors per mouse (palpable in the breast area), and the tumors were measured in 2 dimensions by calipers at the times indicated. The areas of the 2 tumors per mouse were averaged and then these numbers were averaged over all 3 mice per data point and presented as the mean+/-s.e.m. The results are shown in Figure 27. Transfection of the antisense p21 oligonucleotides did not affect growth of Met-1 cells in culture, suggesting an anti-angiogenesis effect *in vivo*.

What is claimed is:

1. A method for inhibiting cyclin-dependent kinase-mediated cell growth and proliferation, comprising inhibiting $p21^{Waf1/Cip1}$ protein with a $p21^{Waf1/Cip1}$ inhibitory agent in an amount effective to inhibit cell growth and proliferation.

2. The method of claim 1, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antisense oligonucleotide molecule directed to the nucleotide sequence of $p21^{Waf1/Cip1}$.

3. The method of claim 1, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antibody directed against the $p21^{Waf1/Cip1}$ protein.

4. The method of claim 3, wherein the antibody is a monoclonal antibody.

5. The method of claim 3, wherein the monoclonal antibody comprises murine antigen binding region residues and human antibody residues.

6. The method of claim 3, wherein the monoclonal antibody is a humanized antibody.

7. The method of claim 3, wherein the monoclonal antibody is a human antibody.

8. A method of inhibiting a disease associated with abnormal cell growth and proliferation, comprising inhibiting $p21^{Waf1/Cip1}$ protein by administering a $p21^{Waf1/Cip1}$ inhibitory agent in an amount effective to inhibit cell growth and proliferation.

9. The method of claim 8, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antisense oligonucleotide molecule directed to the nucleotide sequence of $p21^{Waf1/Cip1}$.

10. The method of claim 8, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antibody directed against the $p21^{Waf1/Cip1}$ protein.

11. The method of claim 10, wherein the antibody is a monoclonal antibody.

12. The method of claim 10, wherein the monoclonal antibody comprises murine antigen binding region residues and human antibody residues.

13. The method of claim 10, wherein the monoclonal antibody is a humanized antibody.

14. The method of claim 10, wherein the monoclonal antibody is a human antibody.

15. The method of claim 8, wherein the disease is a fibrotic disease.

16. The method of claim 15, wherein the disease is selected from the group comprising of atherosclerosis, angioplasty restenosis, and renal mesangial cell proliferation.

17. The method of claim 8, wherein the disease is cancer.

18. The method of claim 8, further comprising exposing the cells to radiation.

19. The method of claim 8, further comprising administering a chemotherapeutic drug.

20. A method of inhibiting angiogenesis, comprising inhibiting $p21^{Waf1/Cip1}$ protein by administering a $p21^{Waf1/Cip1}$ inhibitory agent in an amount effective to inhibit angiogensis in tumors.

21. The method of claim 20, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antisense oligonucleotide molecule directed to the nucleotide sequence of $p21^{Waf1/Cip1}$.

22. The method of claim 20, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antibody directed against the $p21^{Waf1/Cip1}$ protein.

23. The method of claim 22, wherein the antibody is a monoclonal antibody.

24. The method of claim 22, wherein the monoclonal antibody comprises murine antigen binding region residues and human antibody residues.

25. The method of claim 22, wherein the monoclonal antibody is a humanized antibody.

26. The method of claim 22, wherein the monoclonal antibody is a human antibody.

27. A method of inhibiting the growth of tumor cells, comprising administering to a patient an $p21^{Waf1/Cip1}$ inhibitory agent in an amount effective to inhibit growth of the tumor cells.

28. The method of claim 27, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antisense oligonucleotide molecule directed to the nucleotide sequence of $p21^{Waf1/Cip1}$.

29. The method of claim 27, wherein said $p21^{Waf1/Cip1}$ inhibitory agent is an antibody directed against the $p21^{Waf1/Cip1}$ protein.

30. The method of claim 29, wherein the antibody is a monoclonal antibody.

31. The method of claim 29, wherein the monoclonal antibody comprises murine antigen binding region residues and human antibody residues.

32. The method of claim 29, wherein the monoclonal antibody is a humanized antibody.

33. The method of claim 29, wherein the monoclonal antibody is a human antibody.

34. The method of claim 27, further comprising administering a chemotherapeutic drug.

35. The method of claim 27, further comprising administering radiation therapy.

36. A pharmaceutical composition comprising a $p21^{Waf1/Cip1}$ inhibitory agent.

37. The composition of claim 36, wherein said inhibitory agent is an antisense oligonucleotide molecule directed to the nucleotide sequence of $p21^{Waf1/Cip1}$.

38. The composition of claim 36, wherein said inhibitory agent is an antibody directed against the $p21^{Waf1/Cip1}$ protein.

39. The composition of claim 38, wherein said antibody is a monoclonal antibody.

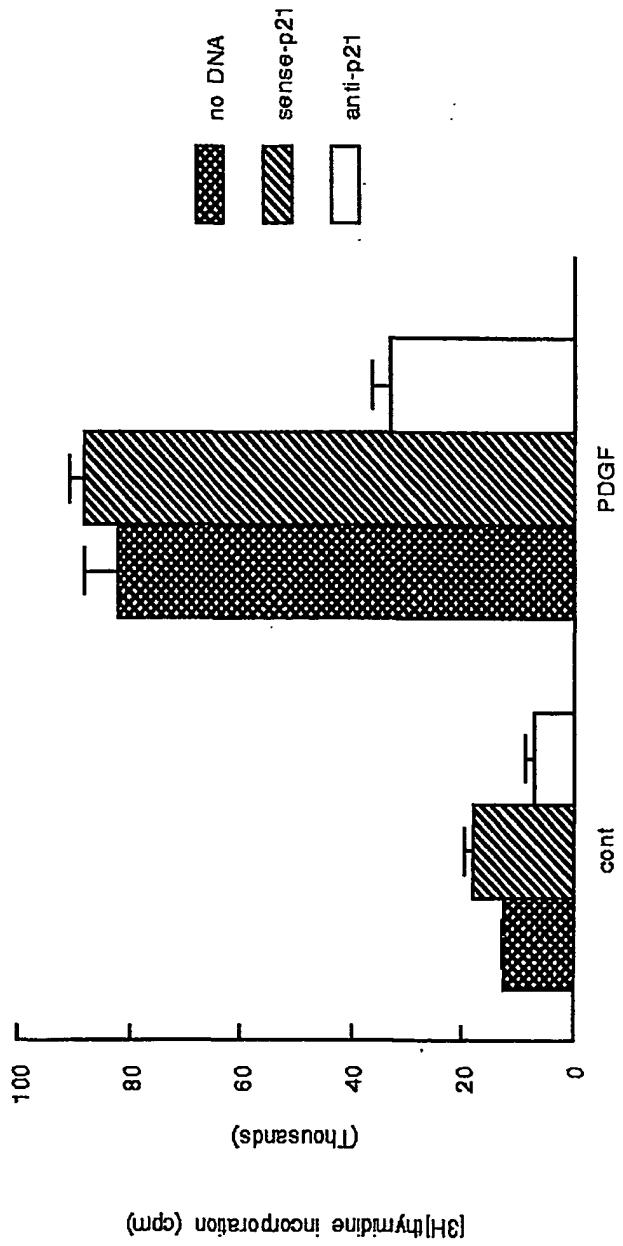
no DNA
sense-p21
anti-p21
PDGF
cont
0
20
40
60
80
100
(Thousands)
[3H]thymidine incorporation (cpm)

FIGURE 1a

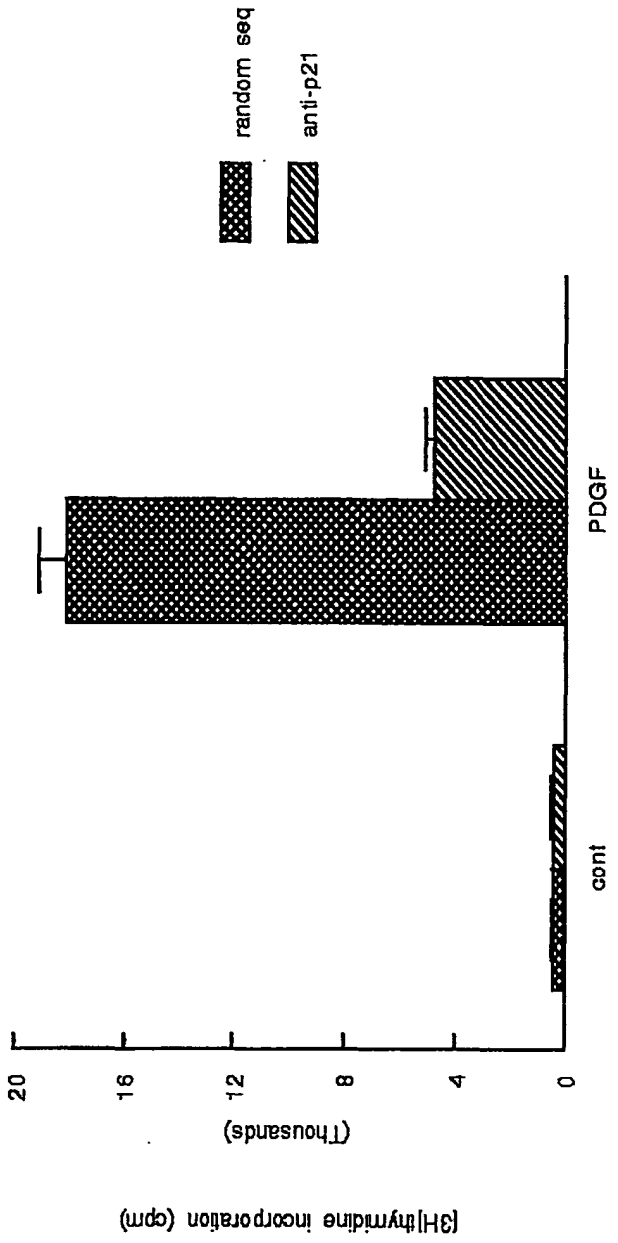
random seq
anti-p21
PDGF
cont
0
4
8
12
16
20
(Thousands)
[3H]thymidine incorporation (cpm)

FIGURE 1b

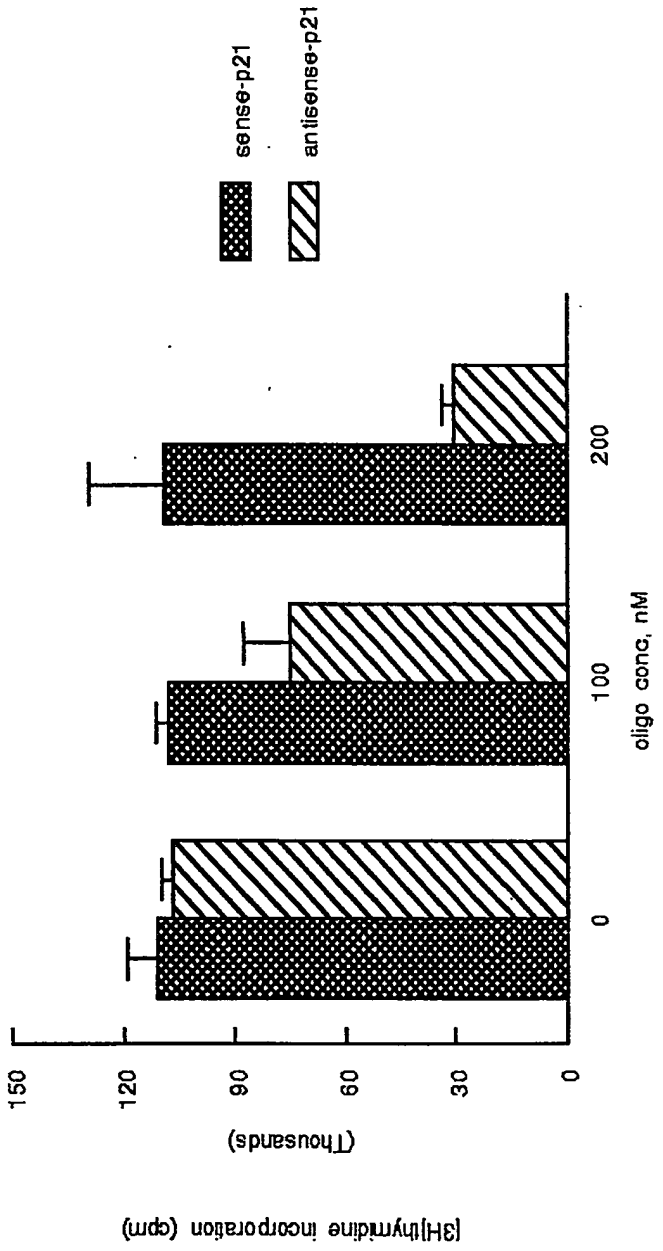
sense-p21
antisense-p21
[3H]thymidine incorporation (cpm)
(Thousands)
150
120
90
60
30
0
0
100
200
oligo conc, nM

FIGURE 1c

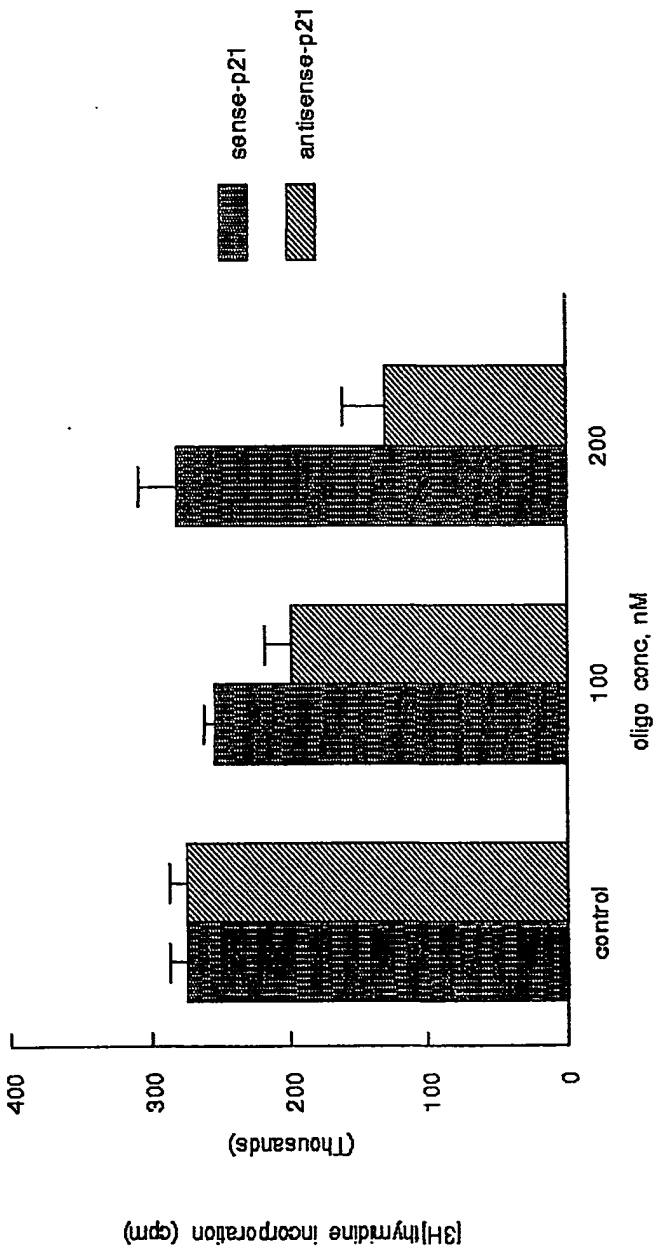
sense-p21
antisense-p21
control
100
200
oligo conc, nM
0
100
200
300
400
(Thousands)
[3H]thymidine incorporation (cpm)

FIGURE 1d

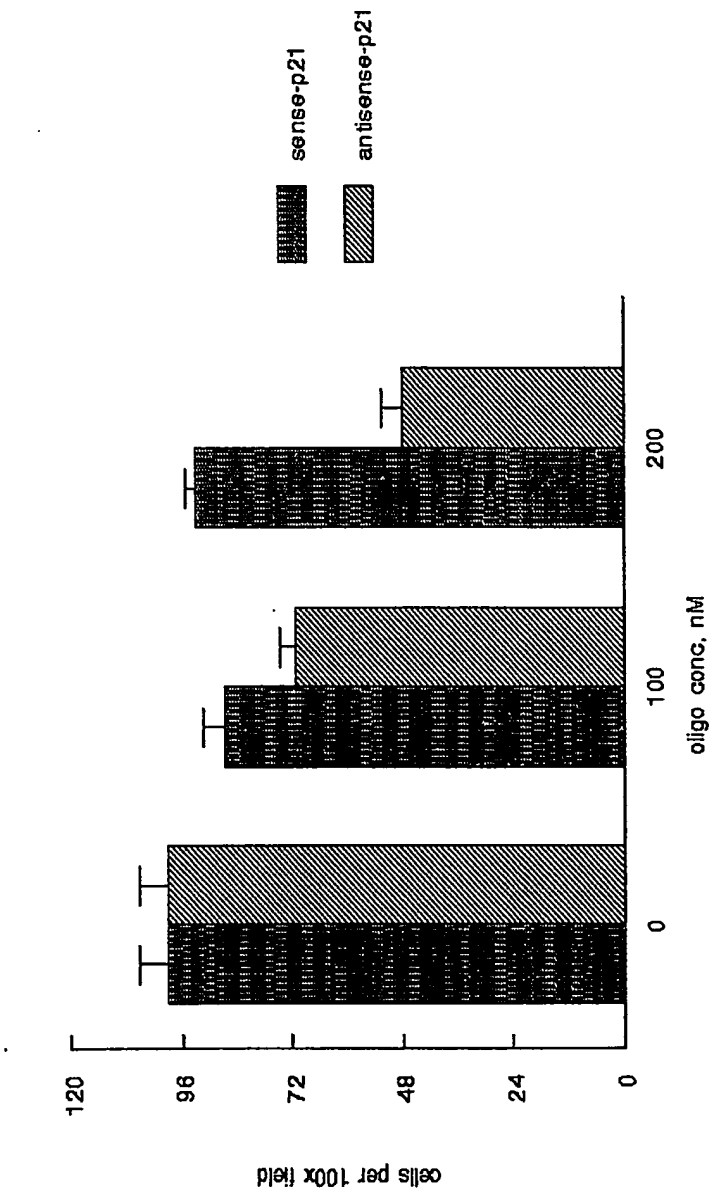
sense-p21
antisense-p21
cells per 100x field
120
96
72
48
24
0
0
100
200
oligo conc, nM

FIGURE 2

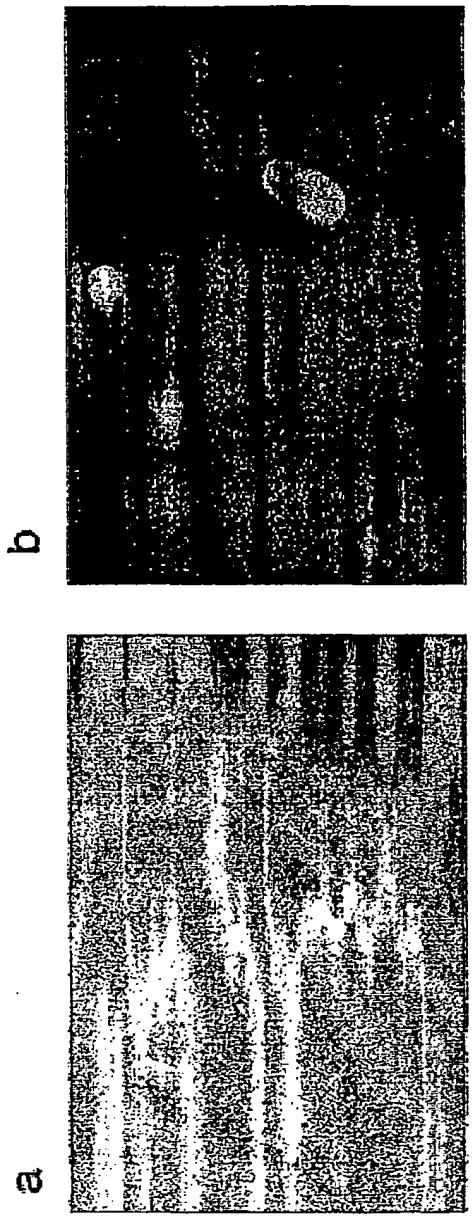


FIGURE 3

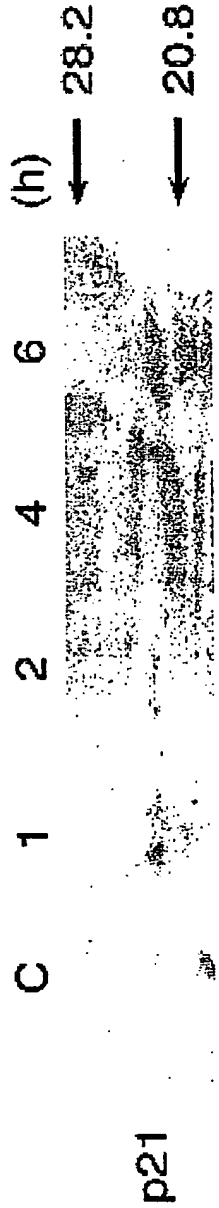


FIGURE 4

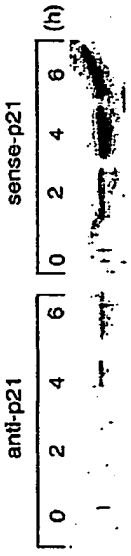


FIGURE 5

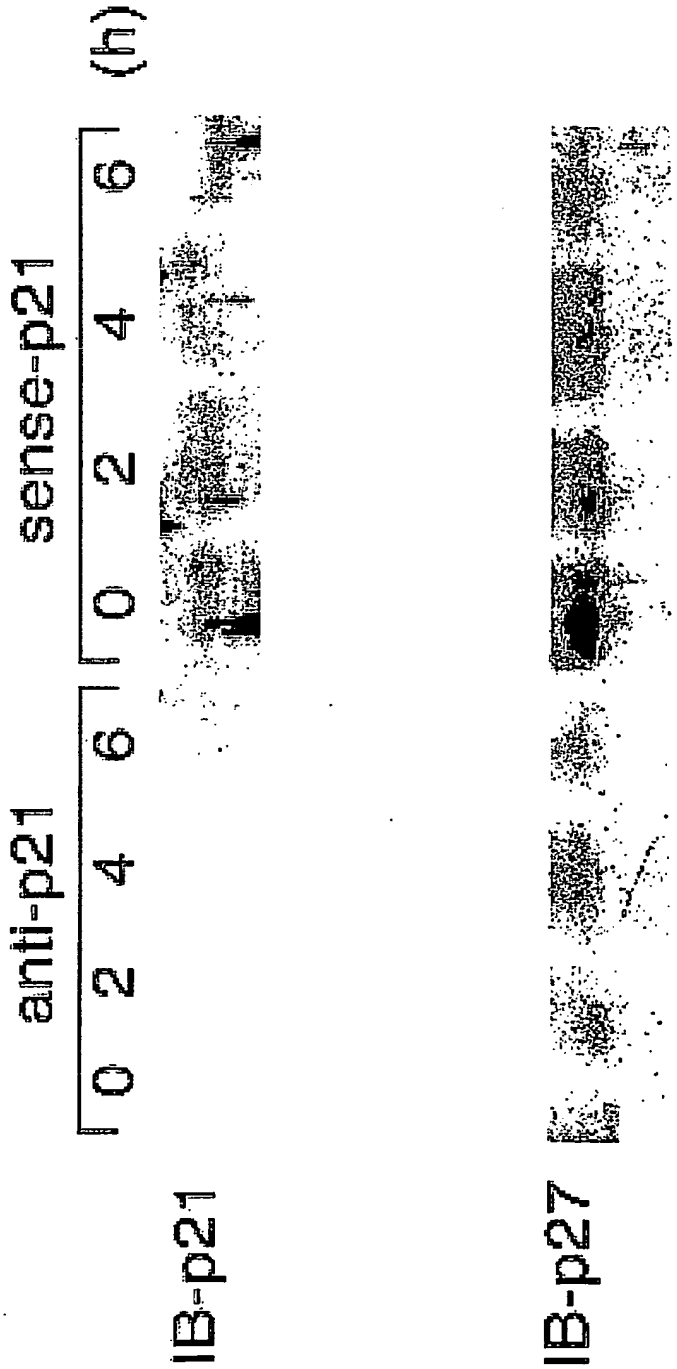


FIGURE 6a

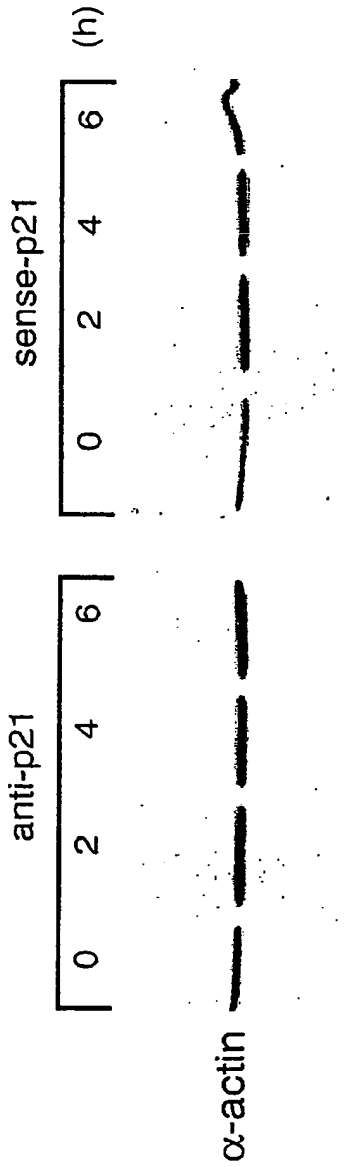


FIGURE 6b

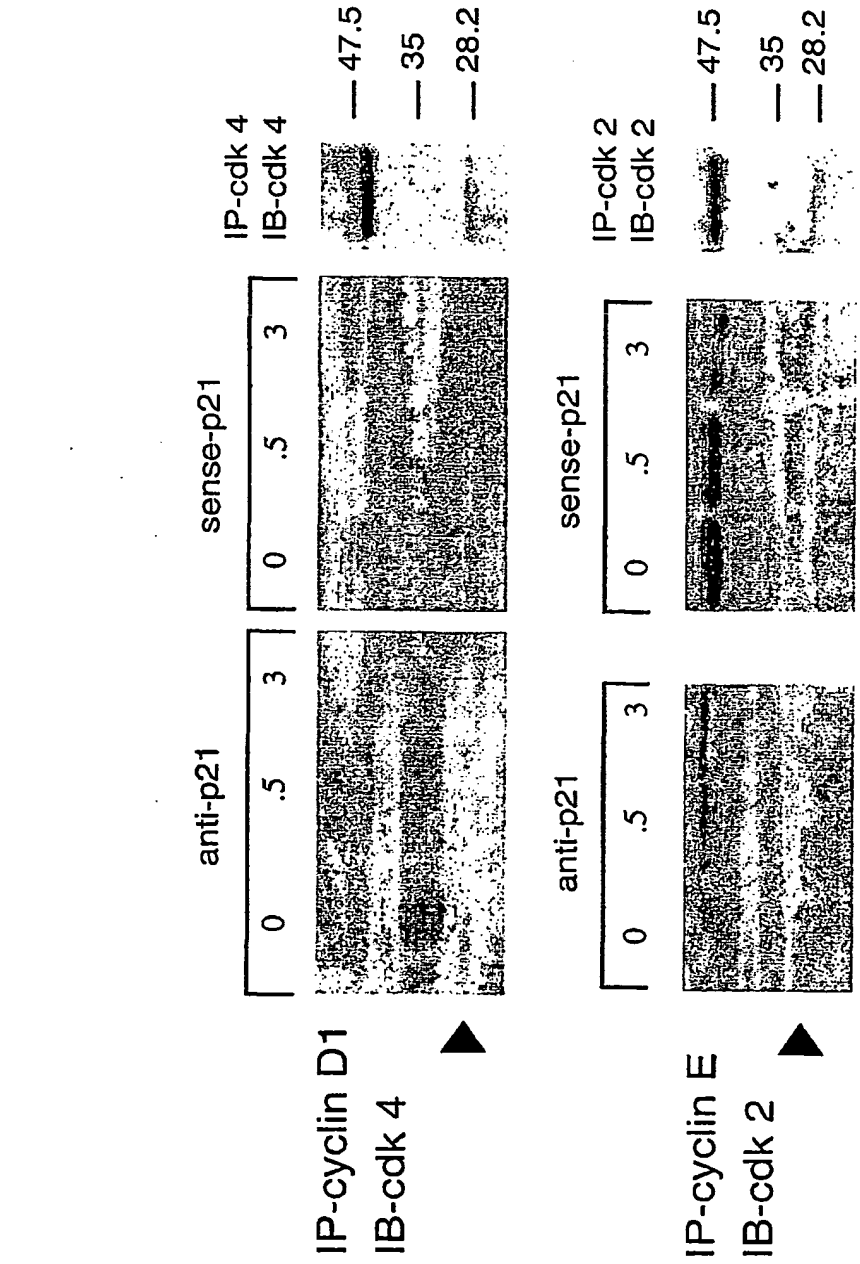
IP-cyclin D1
IB-cdk 4
anti-p21
0 .5 3
sense-p21
0 .5 3
IP-cdk 4
IB-cdk 4
47.5
35
28.2
IP-cyclin E
IB-cdk 2
anti-p21
0 .5 3
sense-p21
0 .5 3
IP-cdk 2
IB-cdk 2
47.5
35
28.2

FIGURE 7

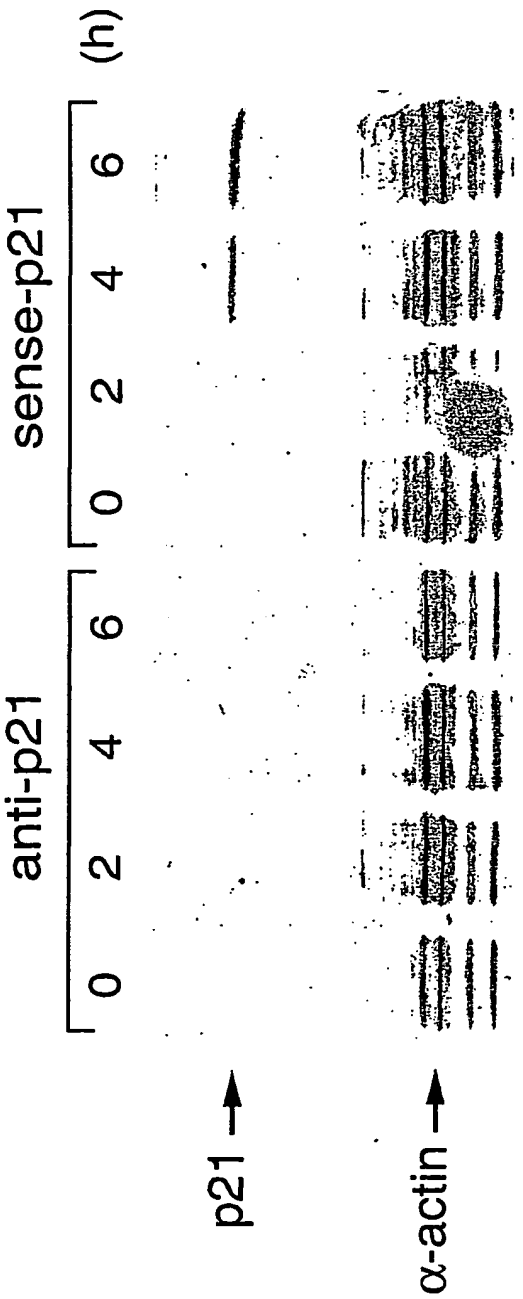


FIGURE 8

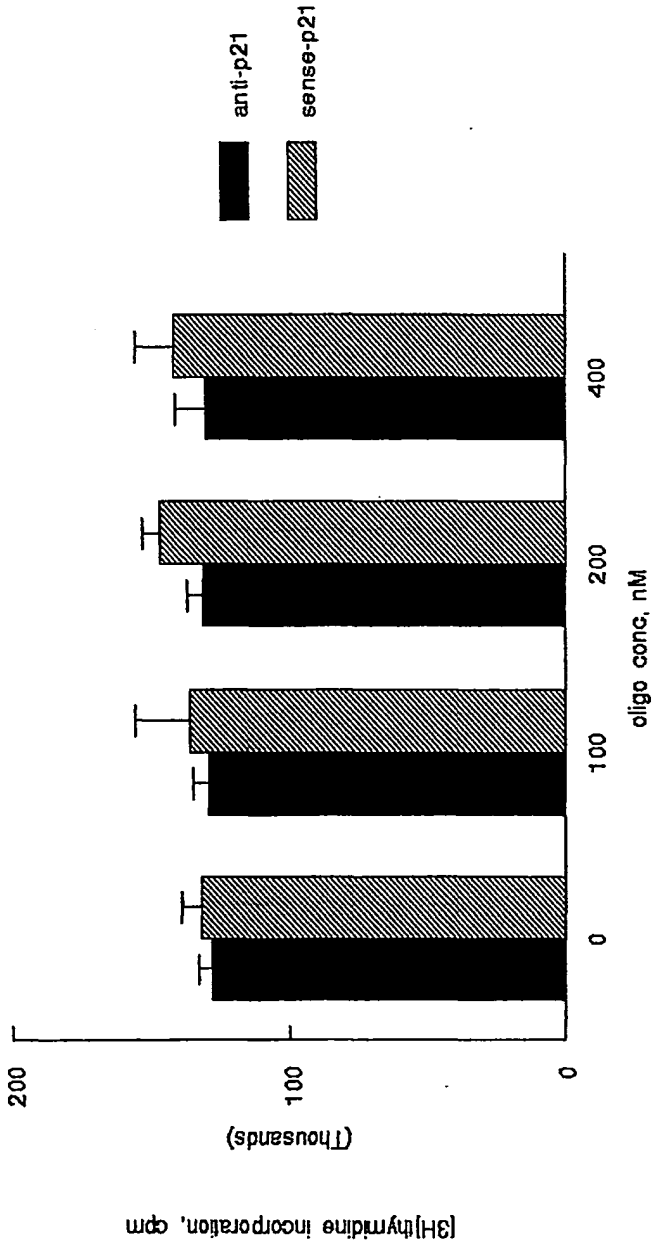
anti-p21
sense-p21
[3H]thymidine incorporation, cpm
(Thousands)
200
100
0
0
100
200
400
oligo conc, nM

FIGURE 9a

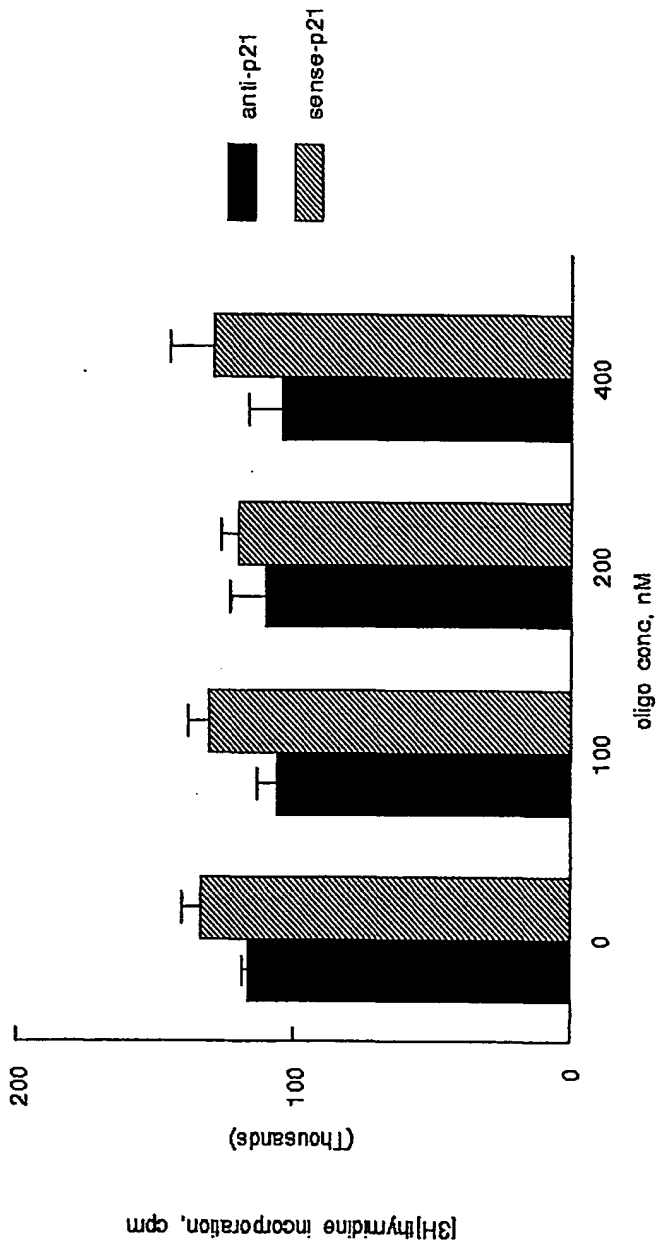
anti-p21
sense-p21
[3H]thymidine incorporation, cpm
(Thousands)
200
100
0
0
100
200
400
oligo conc, nM

FIGURE 9b

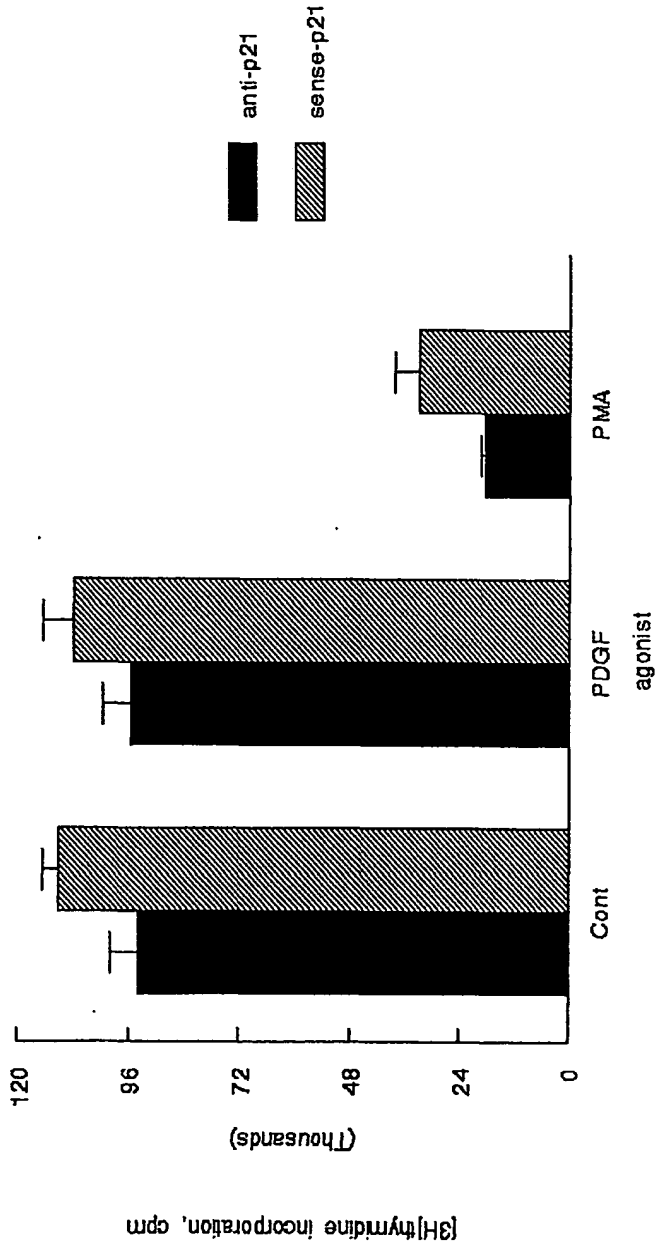
anti-p21
sense-p21
[3H]thymidine incorporation, cpm
(Thousands)
120
96
72
48
24
0
Cont
PDGF
agonist
PMA

FIGURE 9c

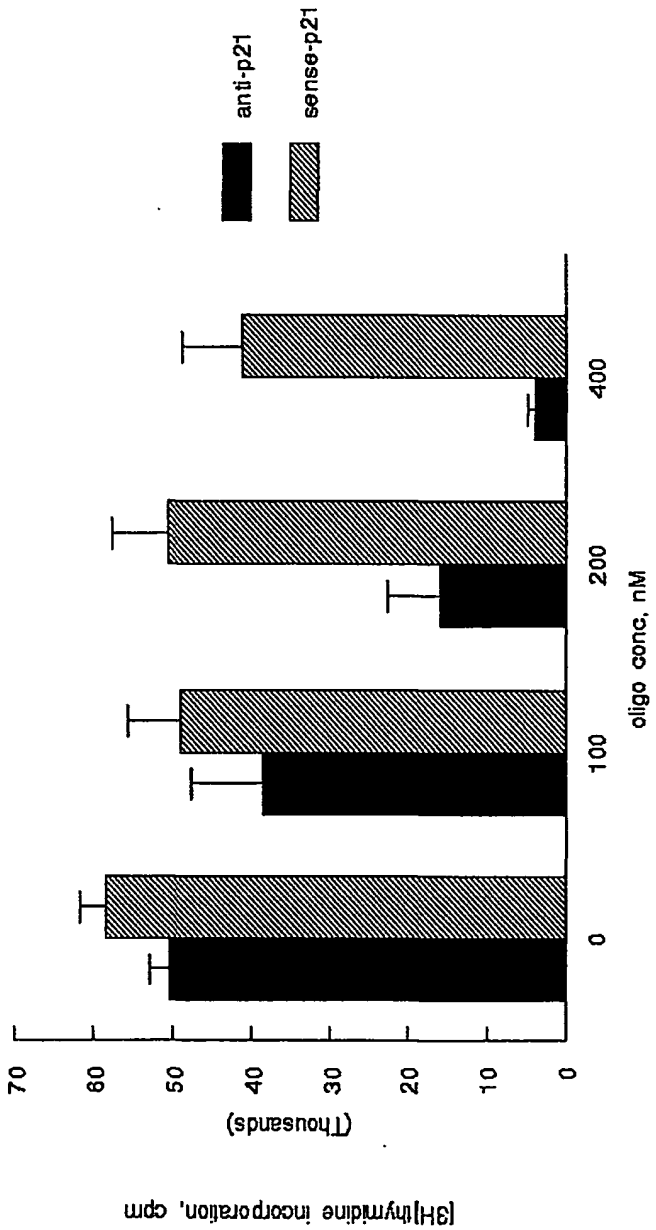
anti-p21
sense-p21
[3H]thymidine incorporation, cpm
(Thousands)
0
10
20
30
40
50
60
70
0
100
200
400
oligo conc, nM

FIGURE 9d

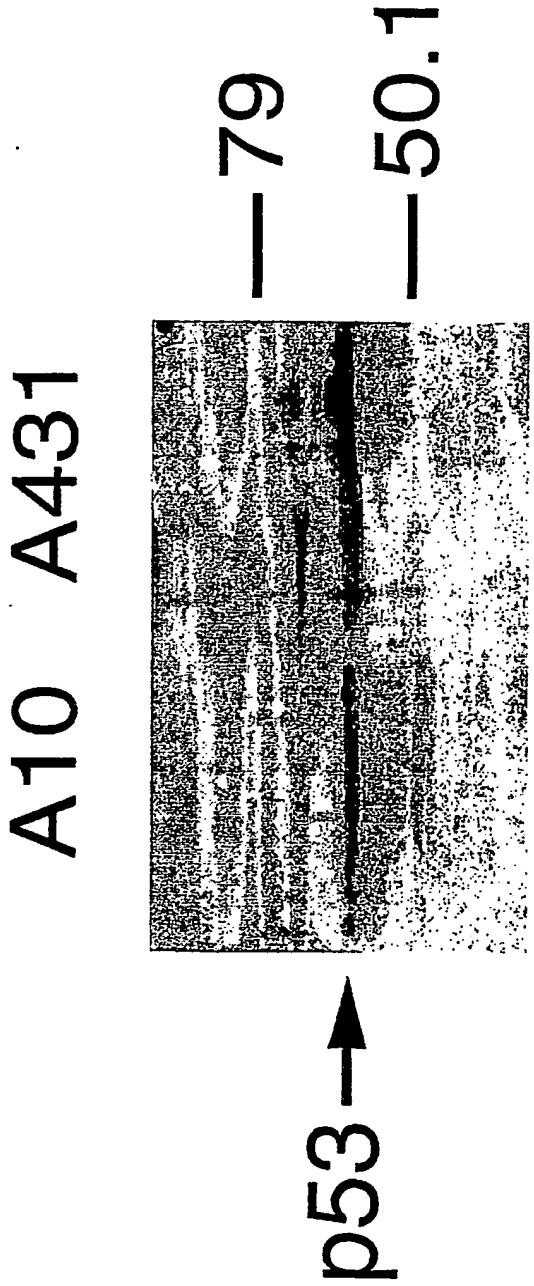


FIGURE 10

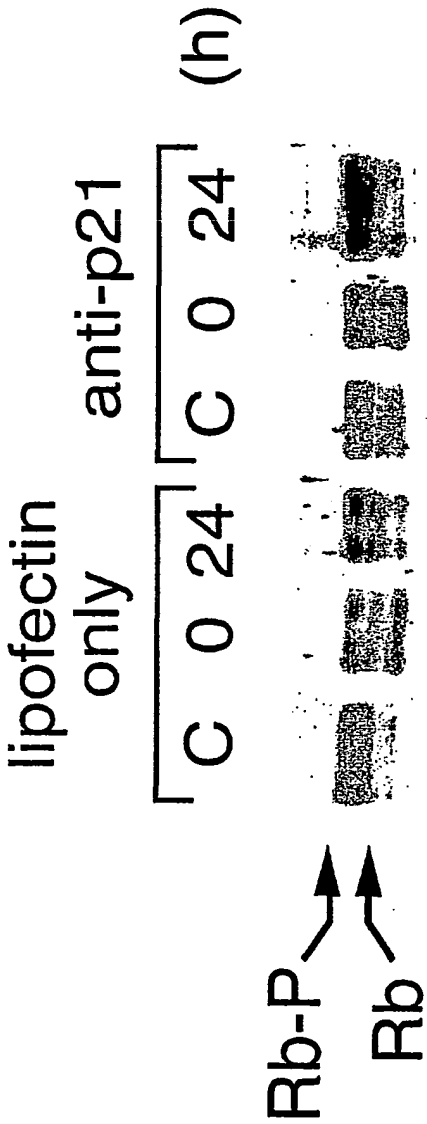


FIGURE 11

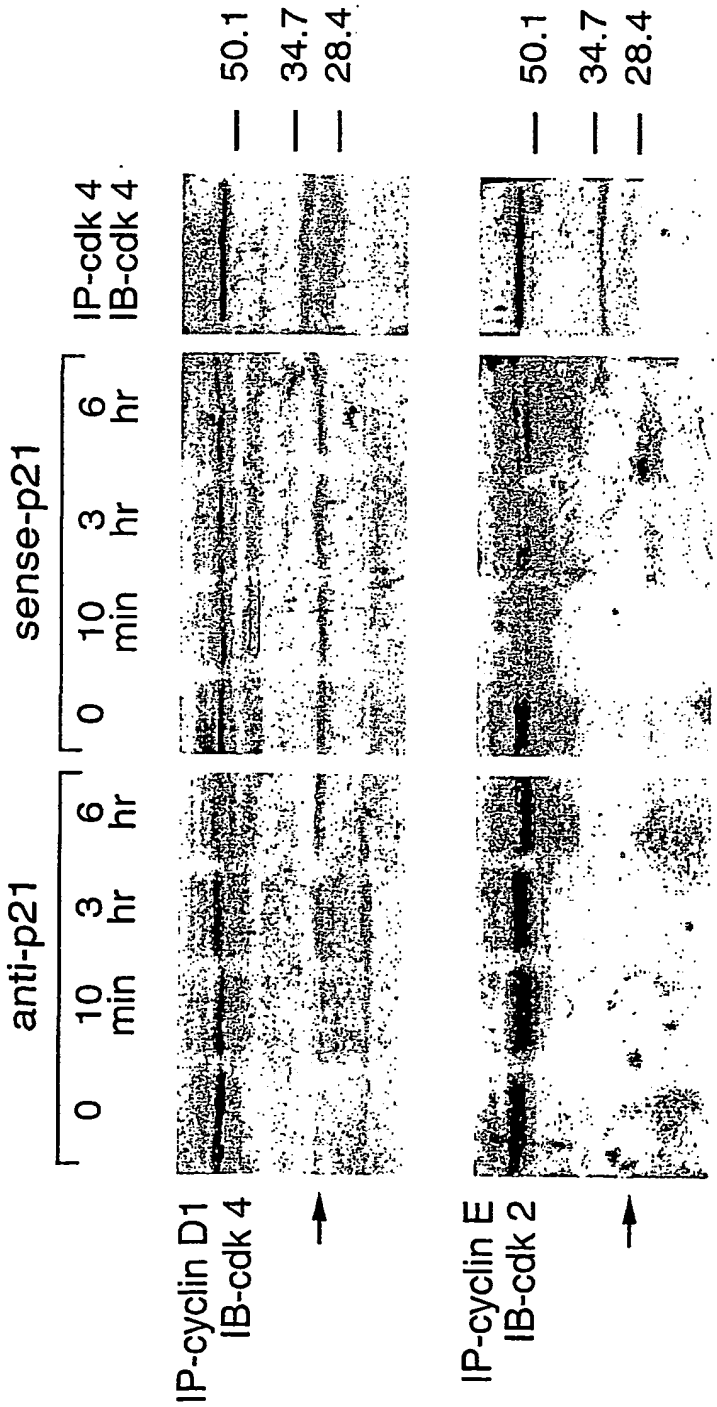
anti-p21
sense-p21
0
10 min
3 hr
6 hr
IP-cyclin D1
IB-cdk 4
IP-cyclin E
IB-cdk 2
IP-cdk 4
IB-cdk 4
50.1
34.7
28.4

FIGURE 12

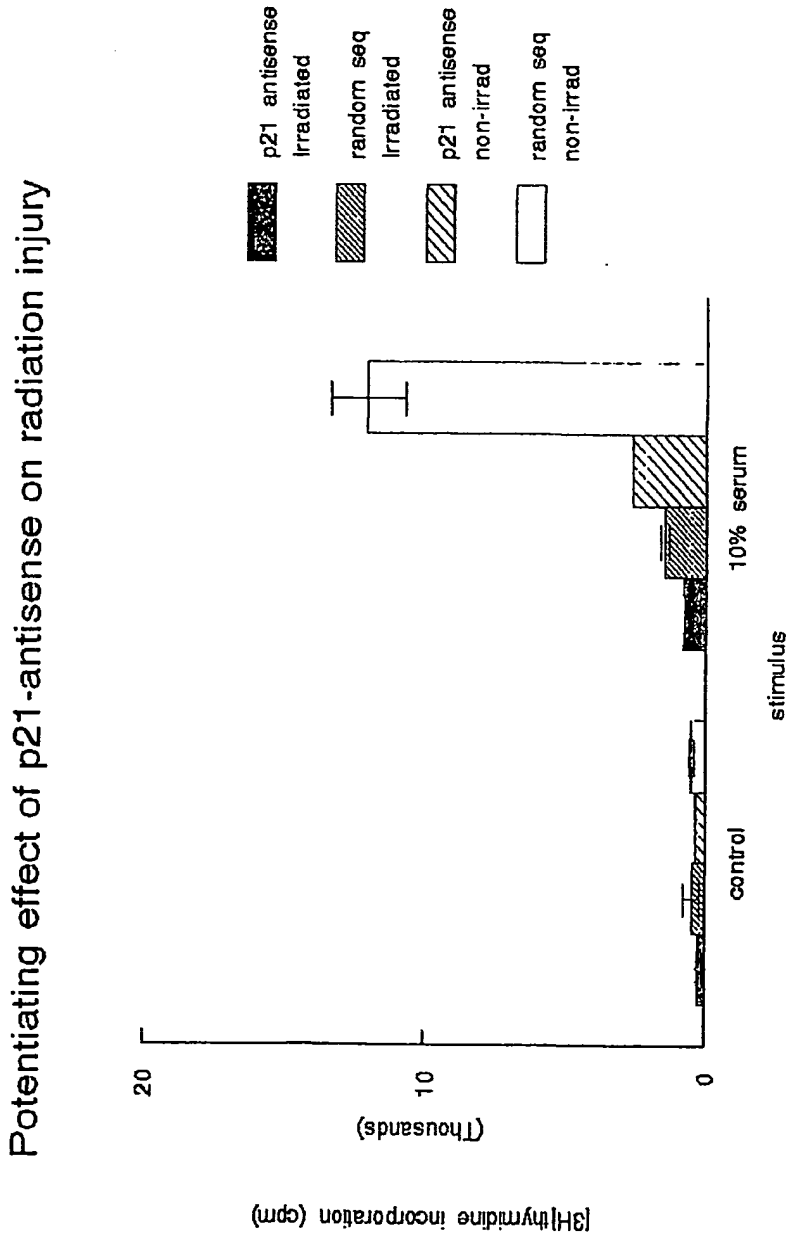
Potentiating effect of p21-antisense on radiation injury
p21 antisense Irradiated
random seq Irradiated
p21 antisense non-irrad
random seq non-irrad
[3H]thymidine incorporation (cpm)
(Thousands)
20
10
0
control
10% serum
stimulus

FIGURE 13

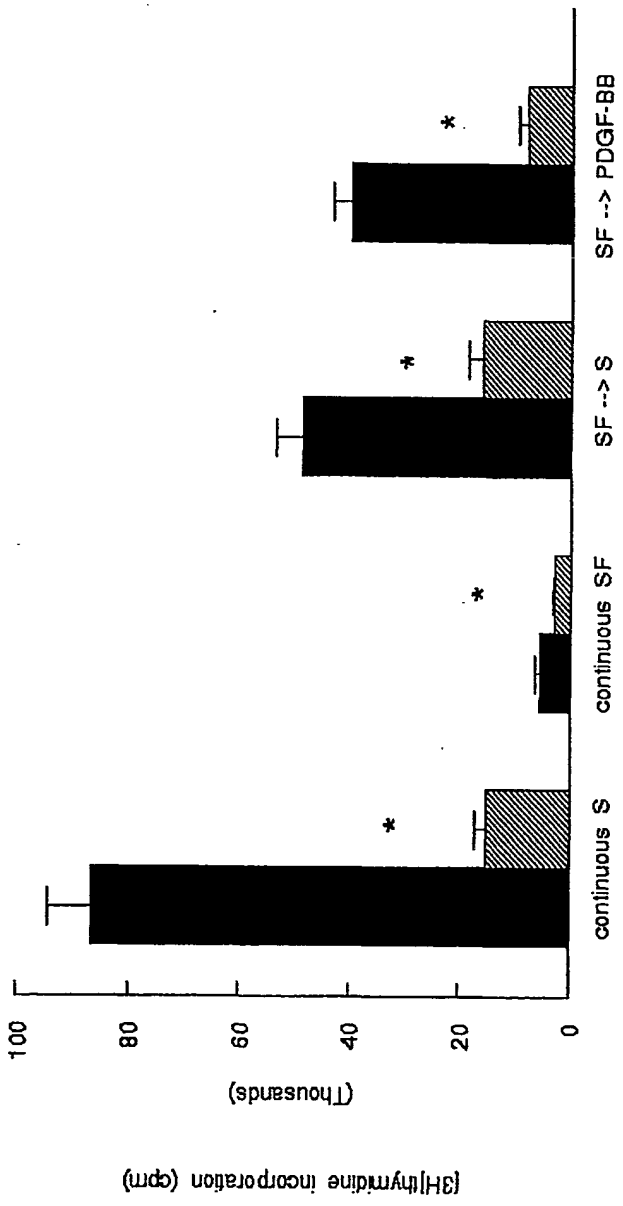
[3H]thymidine incorporation (cpm)
(Thousands)
0
20
40
60
80
100
continuous S
continuous SF
SF --> S
SF --> PDGF-BB
culture condition
*
*
*
*

Figure 14a

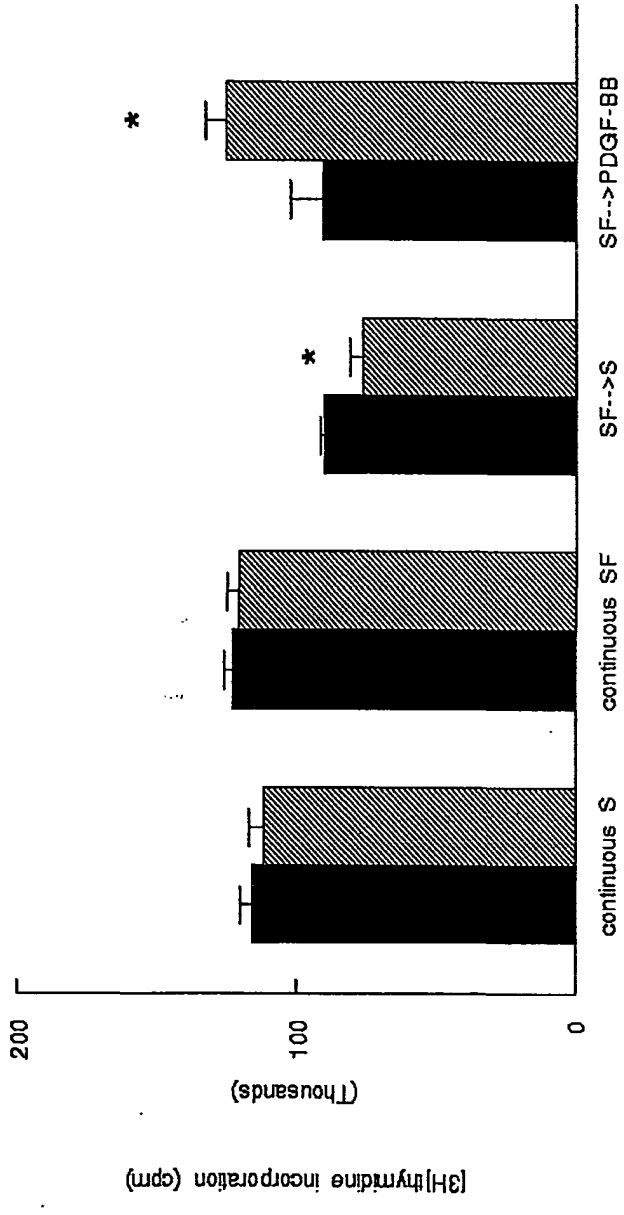
[3H]thymidine incorporation (cpm)
(Thousands)
200
100
0
continuous S
continuous SF
SF-->S
SF-->PDGF-BB
culture condition
*
*

Figure 14b

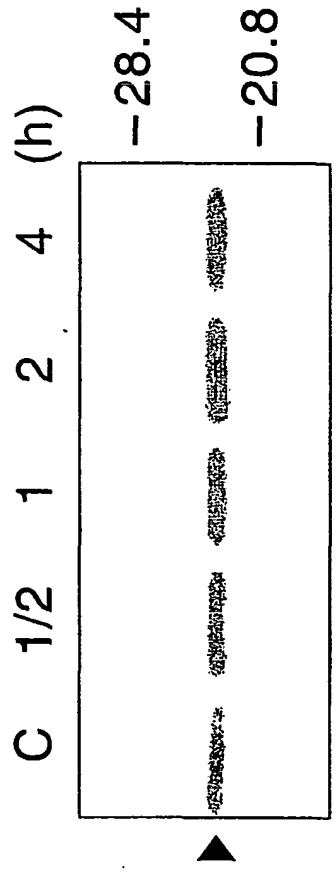


Figure 15a

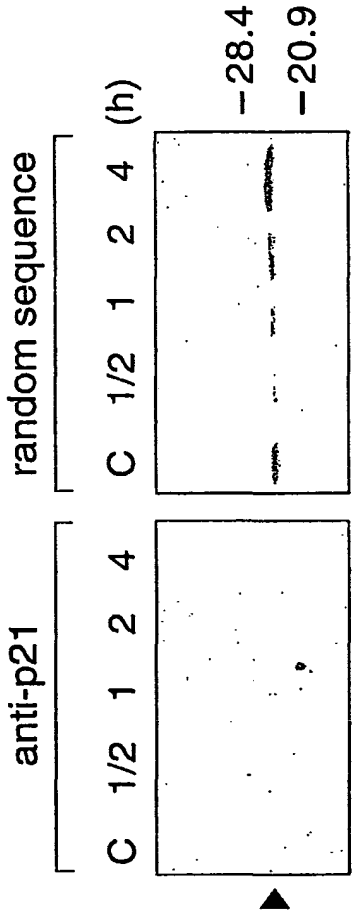


Figure 15b

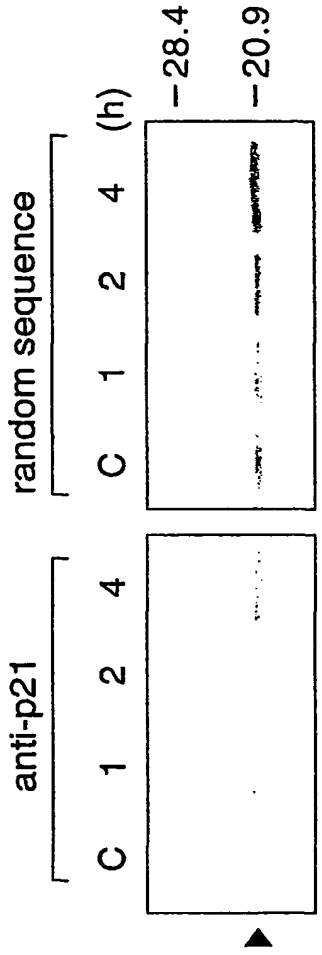


Figure 16

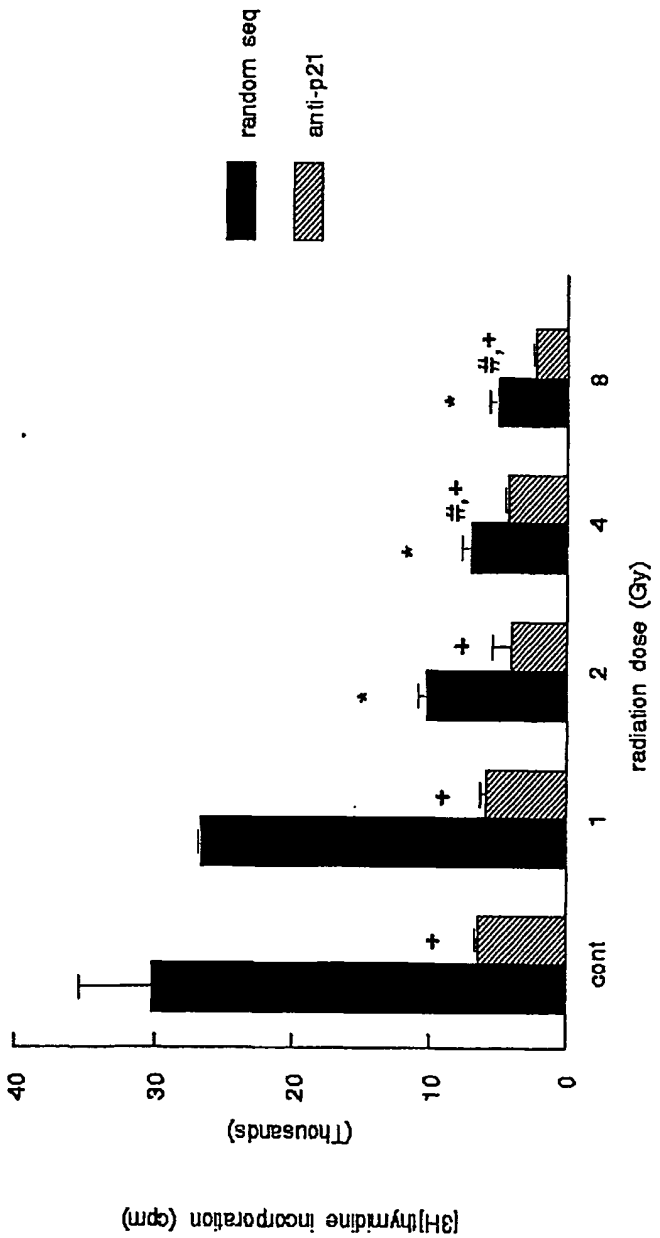
random seq
anti-p21
[3H]thymidine incorporation (cpm)
(Thousands)
0
10
20
30
40
cont
1
2
4
8
radiation dose (Gy)
*
+
#

Figure 17

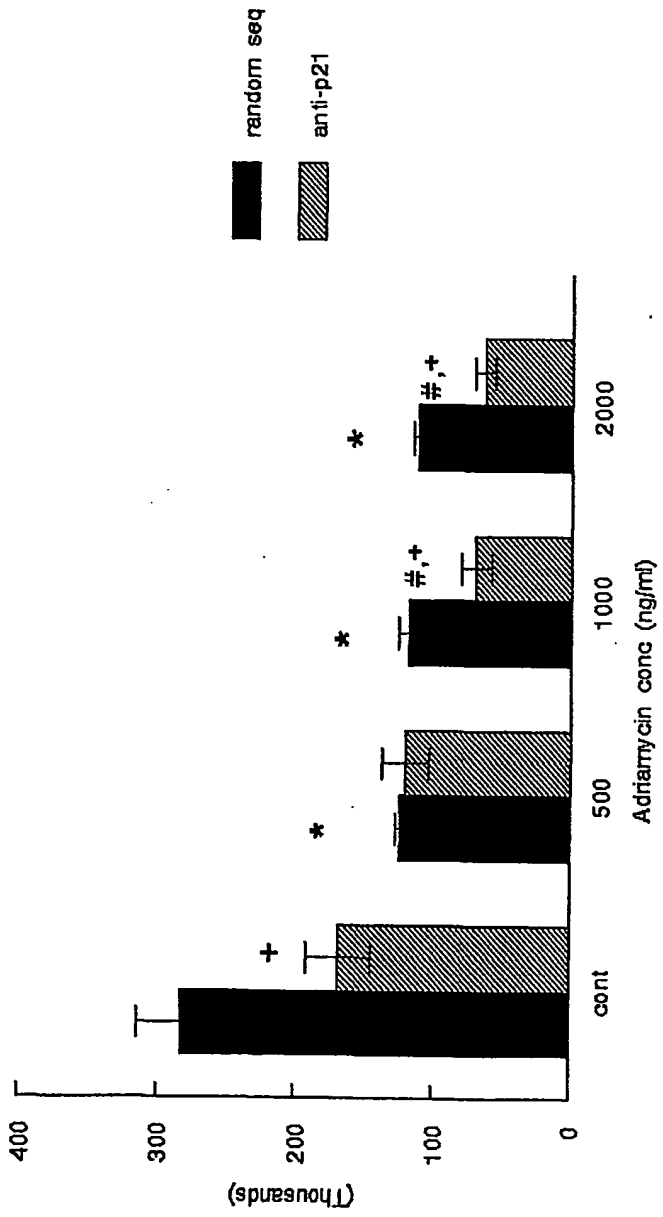
random seq
anti-p21
(Thousands)
400
300
200
100
0
cont
500
1000
2000
Adriamycin conc (ng/ml)
+
*
*
*
#,+
#,+
#,+

Figure 18

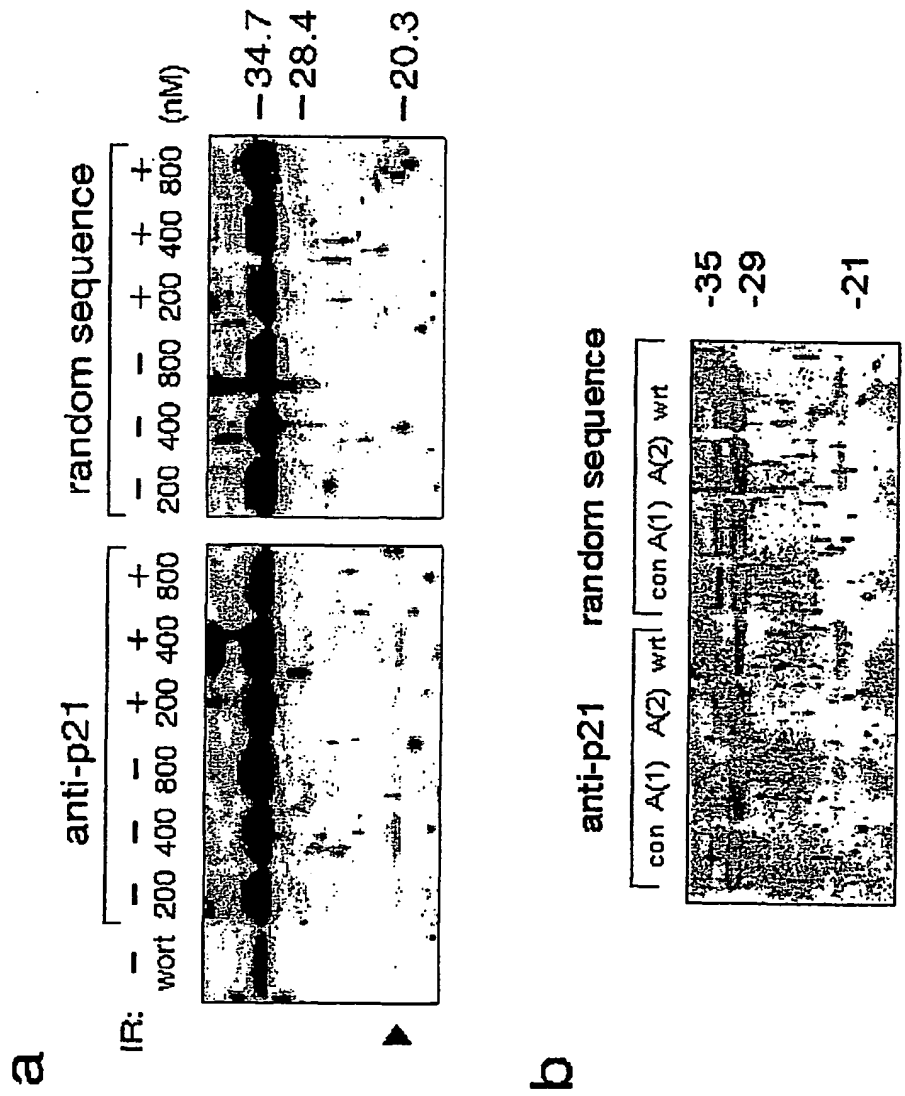
a
anti-p21
random sequence
IR: - - - - + + +
wort 200 400 800 200 400 800 (nM)
- - - + + +
200 400 800 200 400 800
-34.7
-28.4
-20.3
b
anti-p21
random sequence
con A(1) A(2) wrt
con A(1) A(2) wrt
-35
-29
-21

Figure 19

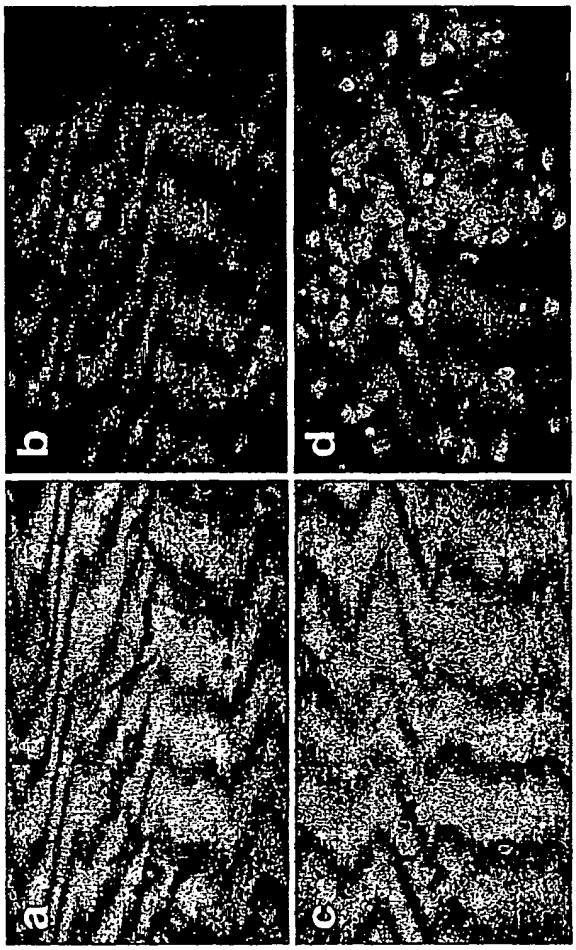


Figure 20

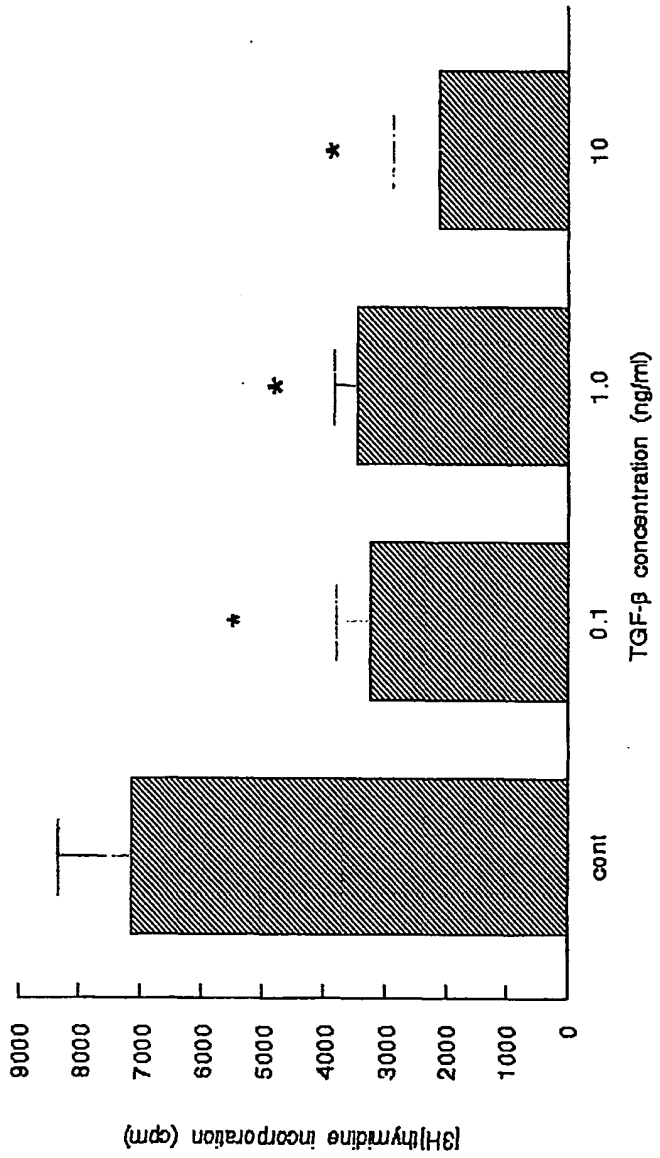
[3H]thymidine incorporation (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cont
0.1
1.0
10
TGF-β concentration (ng/ml)
*
*
*

Figure 21

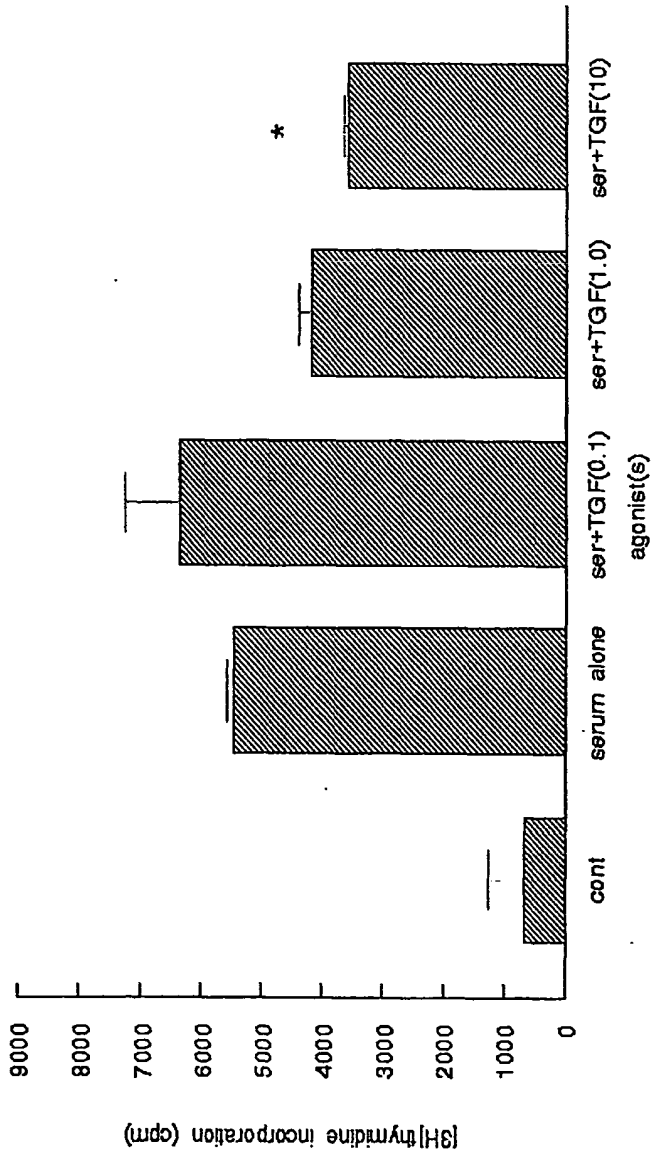
[3H]thymidine incorporation (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cont
serum alone
ser+TGF(0.1)
ser+TGF(1.0)
ser+TGF(10)
agonist(s)
*

Figure 22

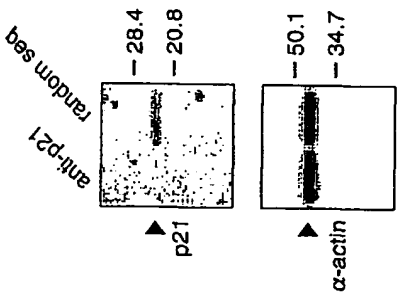


Figure 23

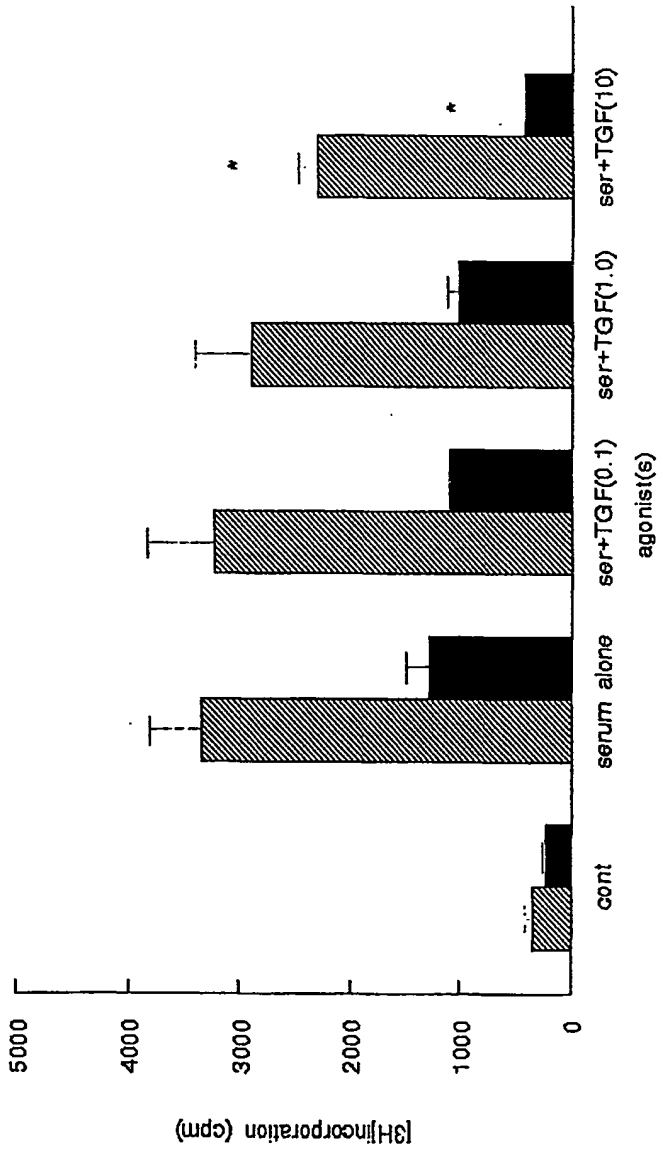
[3H]incorporation (cpm)
0
1000
2000
3000
4000
5000
cont
serum alone
ser+TGF(0.1)
ser+TGF(1.0)
ser+TGF(10)
agonist(s)

Figure 24

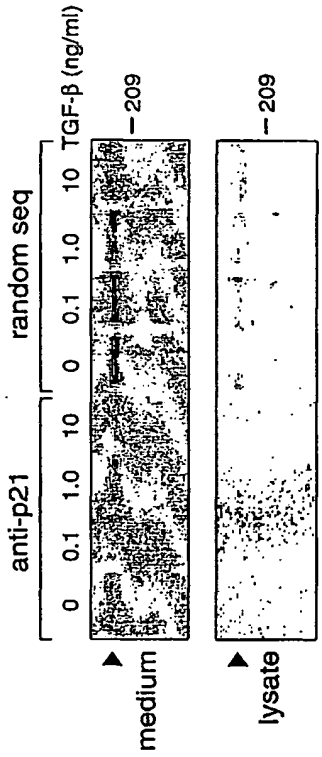
anti-p21
random seq
0 0.1 1.0 10 0 0.1 1.0 10 TGF-β (ng/ml)
209
209
medium
lysate

Figure 25

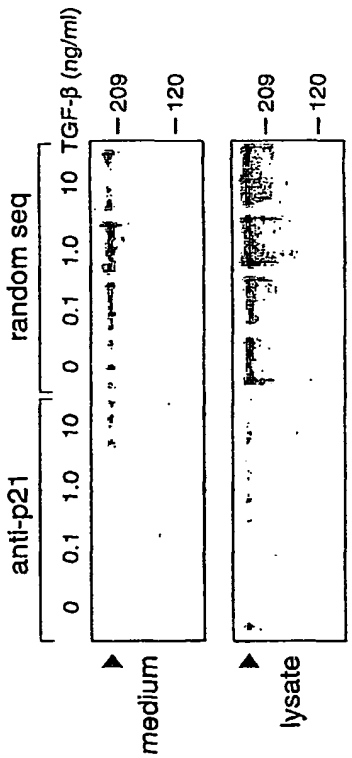
anti-p21
random seq
0
0.1
1.0
10
0
0.1
1.0
10
TGF-β (ng/ml)
209
120
209
120
medium
lysate

Figure 26

SEQUENCE LISTING

<110> The United States of America as represented by the

<120> A NOVEL SPECIFIC INHIBITOR OF THE CYCLIN KINASE INHIBITOR p21Waf1/Cip1 AND METHODS OF USING THE INHIBITOR

<130> 30451.1WO01

<140> Not yet known
<141> 2000-03-29

<150> 60/193,555
<151> 2000-03-29

<160> 3

<170> PatentIn Ver. 2.0

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense p21Waf1/Cip1 oligodeoxynucleotide

<400> 1
gacatcacca ggatcggaca t 21

<210> 2
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense p21Waf1/Cip1 oligodeoxynucleotide

<400> 2
atgtccgatc ctggtgatgt c 21

<210> 3
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: random sequence oligodeoxynucleotide

<400> 3
tggatccgac atgtcaga 18

# INTERNATIONAL SEARCH REPORT

| ational application No. |
|---|
| PCT/US01/10443 |

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE, BIOS, WEST, DERWENT WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,863,904 A (NABEL et al) 26 January 1999, col. 1, lines 10-52, col. 2, lines 1-29, col. 5, lines 15-29. | 15-17 |
| X<br>--<br>Y | WU, M. et al. Roles of the Tumor Suppressor p53 and the Cyclin-dependent Kinase Inhibitor p21 WAF1/CIP1 in Receptor-mediated Apoptosis of WEHI 231 B Lymphoma Cells. J. Exp. Med., 18 May 1998, Vol. 187, No. 10, pages 1671-1679, see entire article. | 1-3, 8-10, 17, 20-22, 27-29, 36-38<br>---<br>4-7, 11-16, 18, 19, 23-26, 30-35, 39 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 14 JUNE 2001 | 30 JUL 2001 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>MARK SHIBUYA |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet) (July 1998) *

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US01/10443

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | OHTSUBO, M. et al. Antisense Oligonucleotide of WAF1 Gene Prevents EGF-induced Cell-cyle Arrest in A431 Cells. Oncogene. 1998, Vol. 16, pages 797-802, see entire article. | 1, 2, 8, 9, 20, 21, 27, 28, 36, 37<br>---<br>15-19, 34, 35 |
| X<br>--<br>Y | MA, Y. et al. Microinjection of Anti-p21 Antibodies Induces Senescent Hs68 Human Fibroblasts to Synthesize DNA but not To Divide. Cancer Research. 15 October 1999, Vol. 59, pages 5341-5348, see entire article. | 1, 3, 8, 10, 17, 20, 22, 27, 29, 36, 38<br>---<br>4-7, 11-16, 18, 19, 23-26, 30-35, 39 |
| Y | WOUTERS, B.G. et al. Loss of p21 Waf1/Cip1 Sensitizes Tumors to Radiation by an Apoptosis-independent Mechanism. Cancer Research. 01 November 1997, Vol. 57, pages 4703-4706, see entire article. | 18, 19, 34, 35 |
| Y | MORRISON, S.L. et al. Chimeric Human Antibody Molecules: Mouse Antigen-Binding Domains with Human Constant Region Domains. Proc. Natl. Acad. Sci. USA. November 1984, Vol. 81, pages 6851-6855, see entire article. | 4-7, 11-14, 23-26, 30-33, 394-7, |

# INTERNATIONAL SEARCH REPORT

national application No.
PCT/US01/10443

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12Q 1/68; G01N 33/53; C12P 19/34; C12N 15/11, 15/63, 15/85; A61K 39/395, 48/00; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 91.1, 194, 320.1, 325, 352, 353, 366, 375; 424/130.1, 133.1, 138.1; 514/44; 530/387.1, 387.3, 387.7; 536/23.1, 24.5

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/6, 7.1, 91.1, 194, 320.1, 325, 352, 353, 366, 375; 424/130.1, 133.1, 138.1; 514/44; 530/387.1, 387.3, 387.7; 536/23.1, 24.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1, 2, 9, 15-21, 27, 28, 34, 35-37, drawn to methods for inhibiting cyclin-dependent kinase functions comprising a p21 Waf1/Cip1 inhibitory agent, wherein said agent is an antisense oligonucleotide directed to the nucleotide sequence of p21 Waf1/Cip1, and pharmaceutical compositions thereof.
Group II, claim(s) 1, 3-20, 22-27, 29-33, 36, 38, 39, drawn to methods for inhibiting cyclin-dependent kinase functions comprising a p21 Waf1/Cip1 inhibitory agent, wherein said agent is an antibody directed to the p21 Waf1/Cip1 protein, and pharmaceutical compositions thereof.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Inhibitory agents directed against p21 Waf1/Cip1 expression and function, including antisense oligonucleotides and antibodies, and methods thereof, are well-known in the prior art, *e.g.*, WU, M. et al., Roles of the Tumor Suppressor p53 and the Cyclin-dependent Kinase Inhibitor p21 WAF1/CIP1 in Receptor-mediated Apoptosis of WEHI 231 B Lymphoma Cells, J. Exp. Med., 18 May 1998, pages 1671-1679. Therefore, there is no special technical features linking the claims.

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] ional application No.
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**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.